Applied and Fundamental Aspects of

Plant Cell, Tissue, and Organ Culture

Edited by
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With 181 Figures

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Preface

Recent progress in the field of plant cell and tissue culture has made this area of research into one of the most dynamic and promising in experimental biology. In vitro cultures are now being used as tools for the study of various basic problems, not only in plant physiology, cell biology and genetics, but also in agriculture, forestry, horticulture and industry. The introduction and development of these techniques has allowed the study of problems previously inaccessible, and has turned the "dreams" of Haberlandt, White and Gautheret into realities.

Cell and tissue cultures have enabled us to increase our knowledge in many areas, including totipotency, differentiation, cell division, cell nutrition, metabolism, radiobiology and cell preservation. We are now able to cultivate cells in quantity, or as clones from single cells, to grow whole plants from isolated meristems, to induce callus or even single cells to develop into complete plants either by organogenesis or directly by embryogenesis in vitro. It is also possible to obtain plants of various levels of ploidy by tissue and endosperm culture, and to produce haploids by using refined embryo culture techniques after interspecific hybridization followed by chromosome elimination of one of the parents. These are only a few from a number of examples which prove the importance of plant cell and tissue culture techniques in research.

Successful work in fundamental research, while being stimulated by applied studies, has also provided the basis for these, and the study of plant tissue and cell culture is no exception to this generalization. For example, it is now possible to propagate plants of economic importance such as orchids and other ornamentals in large numbers by meristem culture or by other in vitro methods and by this means they can be freed from viruses. In plant breeding, embryo, ovary and ovule culture as well as in vitro pollination have been employed to overcome sterility and incompatibility. However, one of the main reasons for the recent increase in the use of plant organs or cells in culture has been the successful production of haploids from anthers or isolated microspores and of protoplasts from higher plant cells, and the recognition of the potential of these materials in genetics and plant breeding.

Haploid plants, especially when they can be produced in large numbers, are important to geneticists because, (a) mutants can be easily detected and, (b) homozygous plants can be obtained directly in a single generation. This material is now available and with anther cultures, or those of isolated microspores it is possible to produce haploids in large numbers from more than 20 species. Protoplasts of higher plant cells are potentially of equal importance as tools for genetic engineering and somatic hybridization. They can be produced by enzyme treatment in large numbers, they can be cultured, they will regenerate cell walls, and

divide and develop into haploid or diploid plants. Under appropriate conditions they fuse and the fusion products can be cultured; even the regeneration of somatic hybrids has been recently reported. Protoplasts can also take up genetic material contained in nuclei and chloroplasts as well as isolated DNA molecules. This provides the opportunity (a) to combine by fusion the genotypes of species which are sexually incompatible and (b) to introduce foreign genetic material such as organelles or DNA into the genome. Since both cultures of haploids and protoplasts can be manipulated by using the methods of microbial genetics it is understandable that these new developments have attracted the intense interest of geneticists and plant breeders.

This survey would be incomplete without a consideration of some of the difficulties inherent in the situation. Cell cultures are being used effectively in vegetative propagation and in the production of virus-free plants as well as in the investigation of secondary products. However, research into the production of haploids and the synthesis of somatic hybrids has not reached a comparable stage of development. The work on haploids has clearly shown that it is mostly microspores from a number of species of the Solanaceae, and some Gramineae, that can be induced either directly to undergo embryogenesis in vitro or indirectly through callus cultures to form plantlets. Similar, but not identical difficulties exist with cultured protoplasts. Despite the fact that the technical hurdles for the production and fusion of protoplasts have been surmounted there are only two reports of successful somatic hybrid formation and here the yields in terms of plantlet formation are far below 1%. Clearly, there are at present restrictions to the application of these techniques and until difficulties have been overcome further progress may be limited. In the case of haploids, conditions must be established for the routine culture of pollen from recalcitrant species and techniques worked out for the selection of induced variability. The problems are similar for the manipulation of protoplasts, more efficient methods must be developed for the growth and selection of hybrid cells and for the regeneration of plantlets from such cultures.

Considering the present situation with its background of success and of unsolved problems, we thought it essential to take a fresh look at the whole topic by producing a book covering the major lines of current research in the subject with the main emphasis on developments relevant to agriculture, forestry, horticulture and industry. This led to the selection of chapters on Regeneration of Plants, Vegetative Propagation and Cloning, Haploids, Cytology, Cytogenetics and Plant Breeding, Protoplasts, Somatic Hybridization and Genetic Engineering, Tissue Culture and Plant Pathology, and Cell Culture and Secondary Products. Some of these chapters are mainly concerned with established technological aspects, while others deal mainly with important theoretical aims and developments for the future. To the latter belong, for instance, articles on gene amplification, incompatibility, and to a lesser degree cell modification and cryobiology, all of which are in *status nascendi.*

The contributions to this book have been written by specialists from different fields and the attempt has been made to avoid unnecessary duplication. However, in certain places repetition does occur and where this may be of benefit to the

reader it has been retained. It is hoped that the efforts of the authors and the editors will provide a book which will be a source of information on current methods, experimental achievements and ideas for a wide range of workers in various disciplines of pure and applied plant science as research workers, teachers or students.

October, 1976

J. Reinert
Y. P. S. Bajaj

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Chapter I

Regeneration of Plants, Vegetative Propagation and Cloning

1. Cell, Tissue and Organ Culture in Sugarcane Improvement[1]

D.J HEINZ, M. KRISHNAMURTHI, L. G. NICKELL, and A. MARETZKI

1. Introduction

Almost 100 million metric tons of sugar or gur was produced worldwide during the crop year 1974–1975. Of this total, 65% was produced from sugarcane. Thus, sugarcane is a major agricultural crop in tropical and subtropical regions of the world and an important export product in many developing countries. These countries account for 70% of the sugar production from sugarcane *(Saccharum officinarum)*.

Sugarcane production offers a continuing challenge to increase sucrose yields and develop disease- and pest-resistant clones.

During the process of evolution, the sugarcane plant has adapted so that it can coexist with other living organisms and with the nonliving environment. Man has continually endeavored to create environments more suitable for his own purposes, and, in this attempt, has hastened the process of positive evolution through artificial selection of plants and animals. This artificial selection involves the creation of variability. Induced variability has been achieved through natural and artificial breeding, and through mutations. Selection is made of those variants adapted for human use. This is known as improvement through "breeding." The aim of any breeding program is the manipulation of genetically variable populations for the identification and selection of desirable characteristics.

Knowledge of the use of plant cell culture techniques has expanded greatly since the demonstration by NICKELL in 1956 that plant materials could be grown in liquid media and treated essentially as microorganisms, and the demonstration by BRAUN in 1959 that an entire plant could be produced from a single cell. BRAUN succeeded because he integrated, in the correct order, a series of steps which he and many other investigators in the field had previously carried out separately. Since then, the totipotency of plant cells has been shown for a number of plants in laboratories throughout the world.

Techniques are now being studied that may have potential in manipulating plant systems at the cellular level for use in "asexual plant improvement". Some species respond readily to one or more of these approaches, while other plants, including sugarcane, present more difficulty.

The purpose of this discussion is to present to the reader advances in plant cell manipulation made with the sugarcane plant.

[1] Work referred to done at the Hawaiian Sugar Planters' Association was supported in part with funds provided by the United States Department of Agriculture Cooperative Agreements Nr. 12-14-5001-83, Nr. 12-14-100-11168(34), and Nr. 12-14-100-10430(34).

1.1 History

Sugarcane tissue and cell culture was initiated for physiological studies at the Experiment Station of the Hawaiian Sugar Planters' Association (HSPA) in 1961 (NICKELL, 1964, 1967). For these studies, mature parenchyma tissue of clone H50-7209 was selected to produce a callus. Material from this original callus was subsequently grown as a suspension culture. Several years later, single cells were isolated from the suspension, and a callus produced from each of them by the nurse culture technique (MUIR *et al.*, 1954). Of five isolated individual cells, each produced tissue differing in appearance. One was white, two beige, and two yellow. The two beige ones grew at different rates, as did the two yellow ones. It appeared there were five different clones. Through cytogenetic studies it was found that each of these subclones had different chromosome numbers (HEINZ *et al.*, 1969).

It was established that the suspension culture from which the explants were made contained cells of varying chromosome numbers. Later, in field-grown plants of the parent variety, it was discovered that individual plants are not made up of cells of uniform chromosome number. In other words, this particular variety exists as a chromosomal mosaic. UKIO URATA at the University of Hawaii (personal communication; URATA and LONG, 1968) had shown totipotency in a number of grasses, including sugarcane, during 1966–67. With this information and the finding of mosaics, a considerable effort at HSPA was started to produce and isolate subclones for improved agronomic characteristics of sugarcane (HEINZ and MEE, 1968, 1969; BARBA and NICKELL, 1969).

About the same time, isolated stem tips were studied in vitro by COLEMAN and NICKELL (1964) to investigate the flowering stimulus in sugarcane. In 1966 researchers at the Agricultural Experiment Station (AES) at Lautoka, Fiji, began work on test tube fertilization of sorghum and sugarcane. Their aim was to preserve florets in cultures so they could be used when desirable male parents flowered. Simultaneously, embryo culture was begun in an effort to overcome embryo-endosperm incompatibility which resulted when interspecific and intergeneric crosses were attempted. The techniques proved most useful (unpublished data).

Soon after HSPA researchers (HEINZ and MEE, 1968, 1969; BARBA and NICKELL, 1969) had conclusively shown totipotency in sugarcane callus cultures, cell and tissue culture work in Fiji was initiated to isolate subclones which would be resistant to Fiji disease (a virus disease transmitted by a hemipteran bug, *Perkinsiella vitiensis*) and to downy mildew, *Sclerospora sacchari*.

Tissue culture work also was begun at the Taiwan Sugar Research Institute (LIU, 1971). COLEMAN, with the US Department of Agriculture, began tissue culture studies in an attempt to isolate mosaic disease-resistant subclones (personal communication). In 1975, workers at the USDA Canal Point Sugarcane Research Station (NORMAN JAMES, personal communication) were successful in differentiating callus and subclones from a number of sugarcane clones.

1.2 Prospective

In addition to sexual breeding techniques, there are many ways of increasing variability. Tissue and cell culture techniques provide many of these new methods for deriving desired genetic variability. Some of the new techniques available are: (1) variation in cell and tissue culture, polyploidy, aneuploidy and chromosomal mosaics; (2) induced mutations; (3) induced polyploidy; (4) haploid plants from pollen; (5) fusion of somatic cells (parasexual hybridization); (6) transformations and (7) culture of embryos resulting from interspecific and intergeneric crosses—the embryos which are incompatible with the endosperm.

While the potential for plant improvement through the above-mentioned methods is great, full exploitation of the system requires precise screening techniques combined with the production of new variants. Until such time, use of these techniques will be exploratory but will offer promise as a tool for use by the plant breeder.

2. Biochemical and Physiological Studies

2.1 Media

The media generally used for the growth of sugarcane cell and tissue cultures are variations of the original WHITE's medium (1943) or the MURASHIGE and SKOOG medium (1962), including the addition of complex materials such as coconut milk, yeast extract, tomato juice, malt extract, etc. Early in the studies with sugarcane cultures, it became evident that WHITE's medium was superior for the growth of sugarcane tissue and cells, but not for totipotency (BARBA and NICKELL, 1969). A chemically defined medium which proved satisfactory for biochemical investigations was developed (NICKELL and MARETZKI, 1969).

In general, sugarcane clones in suspension culture grow better in a medium supplemented with yeast-malt extract than in a medium containing coconut water. A variety of carbohydrates can serve as energy sources, although sucrose and glucose are the outstanding sources (NICKELL and MARETZKI, 1970).

The best medium for callus differentiation and plantlet development (Fig. 1) is that of MURASHIGE and SKOOG (1962) or its modification as shown in Table 1 (HEINZ and MEE, 1969, and unpublished data). Growth in or on White's medium for more than one subculture usually results in a culture which does not differentiate when the auxins are removed (BARBA and NICKELL, 1969). So far, the cause of this loss of ability to differentiate has not been determined.

The presence of an auxin, usually 2,4-dichlorophenoxy acetic acid [2,4-D], but sometimes naphthalene acetic acid [NAA] is necessary for prevention of differentiation of plantlets from callus. Use of indoleacetic acid (IAA) is not desirable, presumably because of the presence of a strong IAA oxidase system in

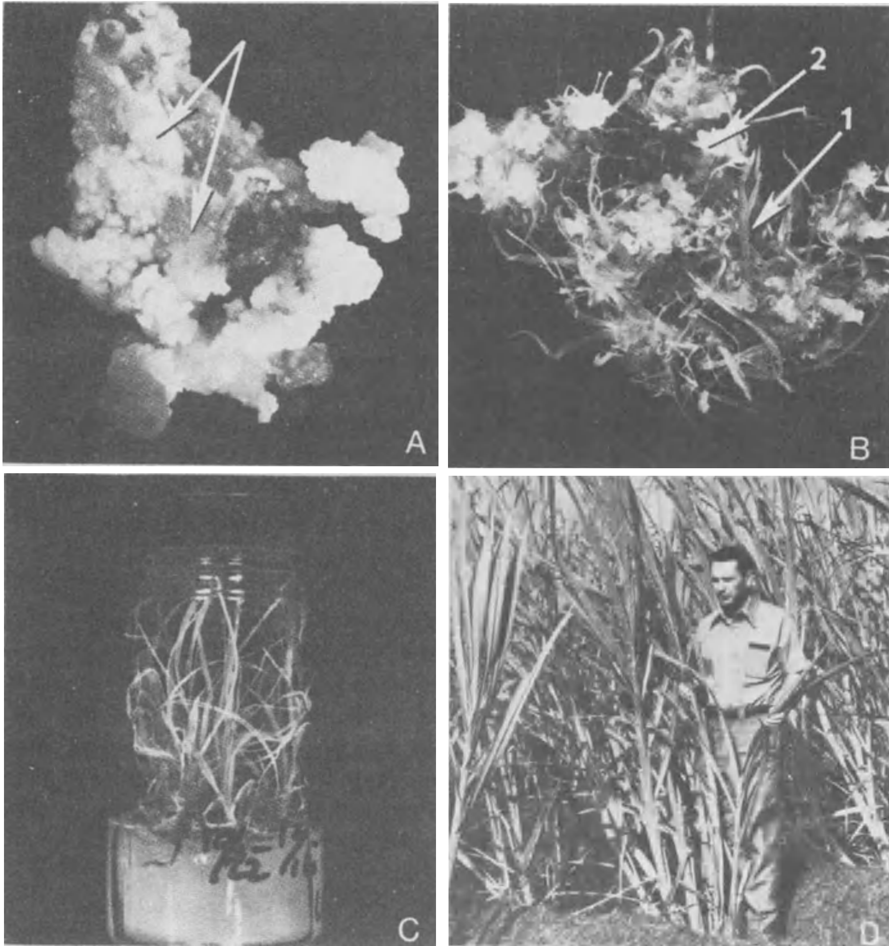


Fig. 1A–D. Stages of development of plantlets derived from callus of sugarcane. (A) Callus mass grown from young leaf tissue (*arrows*) 2x. (B) Plantlet development (*1*) from callus tissue (*2*) 2x. (C) Plantlets ready for transfer from a sterile environment to the field $^1/_2$x. (D) Plants growing in the field prior to selection for desired characters

sugarcane tissues. Although there are varietal differences, callus growth in sugarcane usually proceeds rapidly until there is an apparent exhaustion of some nutrients or until there is desiccation of the medium. For most rapid development, transfer of callus to fresh media is necessary every 3 to 4 weeks.

2.2 Establishing Cultures

The first cultures isolated (NICKELL, 1964) were from the mature parenchyma of internodal tissue. This tissue was used primarily because the internal tissues of the sugarcane stalk are aseptic. Since that time, it has been shown that cultures can be

Table 1. Media used by the Hawaiian Sugar Planters' Association for callus formation and plantlet differentiation

Ingredient	Callus formation Modified Murashige-Skoog (mg/l)	Plantlet development and growth Murashige-Skoog (mg/l)
NH_4NO_3	1650	1650
KNO_3	1900	1900
$CaCl_2 \cdot 2H_2O$	440	440
$MgSO_4 \cdot 7H_2O$	370	370
KH_2PO_4	170	170
Na_2EDTA	37.2	37.2
$FeSO_4 \cdot 7H_2O$	27.8	27.8
H_3BO_3	6.2	6.2
$MnSO_4 \cdot 7H_2O$	22.3	22.3
$ZnSO_4 \cdot 7H_2O$	8.6	8.6
KI	0.83	0.83
$Na_2M_0O_4 \cdot 2H_2O$	0.25	0.25
$CuSO_4 \cdot 5H_2O$	0.025	0.025
$CoCl_2 \cdot 6H_2O$	0.025	0.025
Sucrose	20000	20000
Glycine		2.0
Indoleacetic acid		5.0
Kinetin		2.0
Agar	9000	9000
Myo-Inositol	100	100
Nicotinic acid		0.5
Pyridoxine · HCl		0.5
Thiamine · HCl		0.1
2,4-D	3	
Coconut milk	10% by volume	
pH[a]	5.6	5.6

[a] Adjusted with 1N NaOH.

established from almost any portion of the plant. The most rapid formation of callus and greatest totipotency is obtained from young expanding leaf or young inflorescence tissue.

Many investigators, attempting to establish callus cultures of sugarcane, have found the production and secretion of polyphenols into the medium so great that cultures do not survive, even if they initially become established. Successive, rapid transfers to fresh media at intervals of a day or so can overcome this problem.

2.3 Differentiation

Under proper conditions, the differentiation of either shoots or roots from callus of most sugarcane varieties is easily obtained (HEINZ and MEE, 1969). However, obtaining roots from the differentiated shoots is more difficult. The following series of steps encourages root production: (1) separate the individual plantlets and transfer into fresh agar, (2) after the shoots have developed to a few inches in

height, transfer plantlets to aerated water, (3) transfer plantlets to vermiculite after the initiation of small rootlets, (4) transfer the rooted plants to soil under greenhouse conditions, and (5) transfer to the field. Exact conditions for rooting may have to be modified, depending on the variety of sugarcane.

The failure of callus-derived plantlets to root results in a loss of 20 to 30% of all plants produced. Presently, plants are cultured in water for root development after removal from culture medium. However, recently we have observed that plantlets stored at 15° C develop roots, while plantlets incubated at 10° C, 20° C, and 24° C did not. So far, the phenomenon has been pursued with comparisons of clonal source and different media. Regardless of variety or medium, root formation was highest at 15° C with almost no root development at 10 or 24° C (Heinz and Mee, in prep.). Physiological reasons for this phenomenon have not yet been explored. It is assumed that rooting occurs in response to precise enzyme or hormonal balance which is controlled by temperature. From previous unrelated temperature investigations, we know that sugarcane cell cultures can undergo profound enzymatic changes with temperature modifications of a few degrees (Nickell and Maretzki, 1972).

2.4 Nutrition

2.4.1 Nitrogen

Sugarcane cell suspensions do not grow on nitrate, and grow poorly on an ammonium salt when these are used as sole sources of nitrogen.

Other amino acids, particularly aspartic and glutamic acids, are important sources of nitrogen for sugarcane cell growth. The rate of growth attained by yeast extract cannot be duplicated by a mixture of its amino acid component parts. This indicates a stimulation of growth by yeast extract which is not related to nitrogen metabolism. Nevertheless, it is frequently desirable for metabolic and nutritional investigations to use a fully defined medium instead of yeast extract.

The stimulation of growth of intact plants by arginine (Nickell and Kortschak, 1964) has been duplicated in tissue and cell suspensions (Nickell and Maretzki, 1969). In cell suspensions the presence of arginine reverses the growth-depressing effects of a number of other amino acids. The roles of amino acids, in particular arginine, in regulation of sugarcane cell nutrition has been pursued further (Maretzki *et al.*, 1968, 1969; Maretzki and Thom, 1970).

Recent information suggests that arginine stimulation is characteristic of mature, stationary phase cells. Contrarily, cells undergoing logarithmic or linear growth are not retarded by a lack in exogenously supplied arginine (Glenn, 1973). In mature cells exogenous arginine represses an ornithine carbamyl transferase that appears to be of mitochondrial origin, but does not affect a second, similar enzyme present in the soluble extract (Glenn and Maretzki, in prep.). The possible relationship between these enzymes and growth is being investigated.

The cellular uptake of arginine by suspension culture is rapid. Kinetic measurements show that one site permitting transfer of arginine across the cell mem-

brane is shared by lysine and possibly other amino acids, but also suggests that there is an additional site which is specific for arginine transport (MARETZKI and THOM, 1970).

2.4.2 Carbohydrate

The commercial importance of carbohydrate deposition in sugarcane has helped to draw attention to accumulation, intracellular distribution, and storage physiology in stalk parenchyma tissue (e.g. GLASZIOU and GAYLOR, 1972). Use of cell cultures largely avoids the complications caused by the existence of "free space" in tissue slices.

After cells in suspension culture have passed through the phases of rapid division and enter a stationary phase, sucrose can accumulate to about 10% of the dry weight, presumably in the vacuole. In such cells, glucose, galactose, and fructose at comparable concentrations are accumulated linearly against a concentration gradient over the first 3 to 5 min after contact with the cells (MARETZKI and THOM, 1972a). Characteristic of glucose transport are two distinct rates of substrate transfer, one operative at low exogenous concentrations of the sugar (1–100 μM), the other at high concentrations (above 1 mM). These have been designated as Systems A and B, respectively (MARETZKI and THOM, 1972b). System B transport rates are independent of the developmental stage of the cells, but System A undergoes an almost ten-fold increase in K_m values while the cells are maturing (MARETZKI and THOM, unpublished data).

Sucrose gradient isolation of membrane fractions from cell cultures permits enrichment of plasmalemma vesicles in one of the fractions (38% sucrose) (THOM *et al.*, 1975). Glucose uptake by these vesicles is in accordance with the kinetics of System A, but existence of System B transport in this preparation is still in doubt.

Except by diffusion at high exogenous concentrations, sucrose is unable to penetrate cells in sugarcane tissue without prior hydrolysis. Thus, ^{14}C-sucrose is not detected in cell extracts after cell suspensions are incubated for 5 to 10 min in 5 μM-labeled sucrose.

A cell wall-bound invertase with optimum pH of approximately 5 appears to control the uptake of sucrose. The activity of this enzyme is stimulated by preincubation of the cells in Actinomycin D. This suggests repressor regulation of invertase activity (MARETZKI *et al.*, 1974). Cytoplasmic invertases of sugarcane with activity at either acid or neutral pH have been extensively studied in whole plant tissue. These invertases are also found in cells grown in suspension culture. Continuous culture techniques recently developed will permit maintenance of steady-state cell growth and, as a result, investigations on regulation of invertases and other enzymes.

Sugarcane suspension cultures normally proliferate on galactose for only a limited period of time. Growth ceases by the 4th to 5th day. This pattern can be modified by prolonged incubation of cells on agar in 2% galactose instead of sucrose. Surviving cells are repeatedly subcultured on galactose before a final transfer back to liquid medium. Cell proliferation is slow, but continuous. The cells differ biochemically from the normal type in that they accumulate more glucose-6-phosphate as well as UDP-galactose (MARETZKI and THOM, in prep.).

2.5 Protoplasts

Methods for the isolation and culture of sugarcane protoplasts from suspension cultures have undergone modifications since first described (MARETZKI and NICKELL, 1973) and have been adapted to include leaf apical meristem and root tissues, both at HSPA and AES (KRISHNAMURTHI, in prep.). Protoplast fusion between different sugarcane clones has been explored, using the polyethylene glycol method of KAO and MICHAYLUK (1974). Fusion between *Saccharum officinarum* var. Badila and several clones, including *Saccharum spontaneum* var. Mandaley, *S. spontaneum* var. Tobago and Tobago hybrids, has been successfully completed. Multiple divisions have been observed after fusion of the protoplasts (KRISHNAMURTHI, in prep.).

3. Cytology

3.1 Cytology of Cultured Cells

In our investigations using cell and tissue cultures, it was recognized that considerable variation existed from cell to cell in culture, and among differentiated plantlets. We speculated (NICKELL and HEINZ, 1973) that this variation arose from one or more of the following sources: (1) Inherent variation in the original plant material, i.e. aneuploidy, polyploidy, and chromosome mosaicism. (2) Changes due to exposure of cells to various nutrients, auxins, and other chemicals in the media, causing chromosome breakage, deletions, and mutations. (3) Disruption of intact tissues and organells to form cell suspensions causing mutations.

Cytological examination of clonal material of five *Saccharum* species hybrids and their suspension cultures showed that the chromosome number was stable in four parental clones, but was variable in another (HEINZ *et al.*, 1969). The suspension cultures derived from single cells had been maintained in a yeast extract-enriched nutrient medium for more than six years. These cultures were variable in chromosome number for all clones, and each clone had different chromosome population modes after six years of culture.

One line of variety H 50—7209 had a variation in chromosome number of 71 to 300 with most cells around 80 to 90; a second had 111 to 140; a third ranged from 51 to 185; a fourth had 71 to 90 with most cells being between 71 to 80. The parent clone, H50—7209, has a chromosome number of at least $2n = 108$ to 128. The parent clone of another variety, H49—5, has a chromosome number of $2n = 114$; its culture had a range of $2n = 51$ to 115. Chromosomal mosaicism has been confirmed in sugarcane clones by TLASKAL and HUTCHINSON (1974) and KRISHNAMURTHI and TLASKAL (1974), and HEINZ and MEE (unpublished data) have observed that chromosomal mosaicism in sugarcane is a common occurrence.

The loss of entire chromosomes or deletion of segments or other chromosomal changes will affect the genetic makeup of a cell population. Variations among plantlets derived from such cell populations should prove useful to plant breeders.

3.2 Cytogenetics of Subclones

Subsequent to the recognition of variation in cell populations, HEINZ and MEE (1971) studied two populations derived from callus cultures of sugarcane clones H37—1933 and H50—7209. Variations were observed and studied in morphology, chromosome number, and four enzyme systems. Greater variations in all respects were observed in plantlets derived from H50—7209, known to be a chromosomal mosaic, (HEINZ *et al.*, 1969) than from plantlets derived from H37—1933, considered to be chromosomally stable. Of the 37 plantlets of H50—7209, all but one had cell-to-cell variation in chromosome number. In all but one of these 36 variable plants, the over-all range in chromosome number was 2n = 94 to 120. The exceptional plant had a range of 2n = 17 to 118. Among 8 plants of H37—1933, all had 2n = ca. 106. HEINZ and MEE (1971) presented evidence to show that part of the chromosomal variation was due to the asynchronous division of multinucleate cells. Variation in chromosome number had been observed also in intact plants both at meiosis and mitosis.

We have observed that cell cultures form variation in chromosome numbers at rates greater than in intact plants. This phenomenon is probably due to a better chance for survival of highly variant cells in vitro than in vivo. Subclones thus possessing varied chromosome numbers, differ in phenotypic expression ranging from normal to plants which are dwarf and lack vigor. The isolated plantlets when grown in the field remain stable, and do not produce sectors or chimeras; although there are differences among plantlets.

3.3 Polyploidy

Attempts have been made over the years (GRASSL, 1966; JAGATHESAN, 1966) to double the chromosome number of sugarcane. Investigators at the British West Indies Central Cane Breeding Station (SISODIA, 1965, 1966; PHILLIPS, 1967) succeeded in doubling the chromosome number in three seedlings of *S. spontaneum* from treated seed. Repeated attempts failed with other clones using both seed and lateral buds.

By treating cell suspensions with colchicine (50 mg/l), the chromosome number of sugarcane cells has been doubled (MEE *et al.*, 1969). Later, we doubled the chromosome number in plants of a commercial clone through doubled cell cultures (HEINZ and MEE, 1970). Subsequently, we have developed plantlets with a doubled chromosome number from over 100 clones representing commercial, intra, and interspecific hybrid clones (unpublished data).

Plantlets having a doubled chromosome number developed from commercial clones were stunted, lacked vigor, and with two exceptions, failed to flower. Some doubled plants, developed from intra and interspecific hybrids, have shown vigor and have flowered. These plants should be valuable in the breeding program.

3.4 Haploidy

In sugarcane, as in other crop plants, availability of haploid plants is a desirable objective. If commercial sugarcane represents the high ploidy it is reported to,

chromosomal redundancy seems probable. A reduction in genes controlling a given biochemical pathway may prove helpful for metabolic investigations. Haploids should also assist the selection of genetic markers for proof in parasexual hybridization. The genetic complexity of sugarcane hybrids presently prevents the development of isogenic lines of sugarcane. Culture of anthers can yield haploid tissue or plants or can be doubled to yield homozygous diploids. In sugarcane, haploid cells or plants have not been achieved by any of several experimental approaches. Several difficulties have contributed to the lack of success including seasonal flowering of sugarcane, brief longevity of cane pollen, choice of the proper stage of pollen development, and the mechanical difficulties that attend isolation of large numbers of anthers, 2 to 3 mm in length. Nevertheless, some progress has been made. Sustained longevity of the immature pollen requires high sucrose concentrations (15%) and apparently a very specific stage of pollen development. Formation of multiple-celled pollen grains, up to 10 divisions, was observed in anthers incubated for 2 weeks on a medium containing coconut milk without supplemental auxin or kinetin (MOORE *et al.*, unpublished results).

4. Mutagenics

4.1 Disease Resistance

To date, the most promising use of sugarcane cell cultures for crop improvement is in the development of subclones of commercial clones deficient in one or two desirable characteristics, especially disease resistance. We (HEINZ, 1973 b) reported the development of eyespot-resistant subclones from a susceptible parent clone. KRISHNAMURTHI and TLASKAL (1974) have reported the development of resistant subclones from Fiji and downy mildew-susceptible parent clones.

4.1.1 Eyespot Disease

Eyespot disease of sugarcane is caused by *Helminthosporium sacchari*, which produces a toxin, helminthosporoside (STEINER and BYTHER, 1971; STEINER and STROBEL, 1971). The disease is characterized by a lesion in leaf tissue with streaks or runners extending from the primary infection towards the tip of the leaf. Due to high genetic resistance to this pathogen, it does not present a serious problem, but is useful in the study of variation in plantlets derived from cell cultures.

In a recent study (HEINZ *et al.*, in prep.), suspension cultures of the susceptible clone CP57-603 (rated 6 on a scale of 1–9, 1 being resistant, 9 susceptible) were used in an attempt to screen for resistance in vitro using helminthosporoside. Suspension cultures were treated with a chemical mutagen, methyl methanesulphonate, ionizing radiation, or were left untreated and subsequently cultured in vitro in 0, 1, or 5% helminthosporoside (crude extract). Plantlets were differentiated, planted in the field, and screened for resistance with helminthosporoside when the plantlets were mature.

Presented in Table 2 are partial results of this study. Based on disease susceptibility, all subclones rated 4 or lower would be acceptable for commercial planting.

Table 2. Reaction of CP57–603 callus subclones to eyespot disease

Treatment	Helmintho-sporoside %	Number of subclones	Disease rating[a] % of subclones						
			9–8	7	6	5	4	3	2
Check CP57–603					100				
Control	0	63	16	21	24	8	11	9	11
Control	5	87	4	6	30	34	13	3	10
MMS 50 mg/l		99	2	14	35	35	8	3	3
MMS 50 mg/l	5	81	2	20	20	20	5	20	13
Ionizing radiation 3 KR	0	57	10	21	11	23	12	11	12

[a] Rating based on scale of 1–9; 1 being resistant, and 9 susceptible.

Many of the resistant subclones are not visually different from the parent clone; these will be placed in a yield trial.

We were hoping to screen out all susceptible cells in vitro through the use of helminthosporoside; the data suggest this did not occur. Failure to produce an increased percentage of resistant plantlets when intact cells or clumps of cells were exposed to helminthosporoside may be because not all cells were exposed to the toxin. We contemplate treating single-cell suspensions to see if this will provide better in vitro screening for resistant cells. However, the frequency of resistant subclones obtained without the aid of in vitro selection warrants further studies with other diseases.

4.1.2 Fiji Disease

It is suspected that Fiji disease of sugarcane is caused by a virus. The disease is detected by the presence of galls on leaves as a result of the extensive proliferation of phloem tissue. Once a plant is infected, it becomes stunted, and in advanced cases, the spindle dies. The virus is transmitted by a hemipteran leafhopper, *Perkinsiella vitisensis Kirk.* Details on transmission, disease development, and systems used for grading susceptibility have been reported previously (Husain *et al.*, 1967; Hutchinson *et al.*, 1970).

Development of Fiji-resistant subclones from susceptible parents has been carried out in Fiji with the susceptible clone, Pindar. Thirty-eight subclones were developed from tissue culture and tested for reaction to Fiji disease. Four subclones were resistant and one had intermediate resistance. The resistant subclones were tested over a number of years in several locations and have maintained resistance. Since the work began, subclones with a wide range reaction to the disease have been developed from a number of clones (Table 3).

The Fiji disease-resistant Pindar subclones have been screened for yield potential. Presented in Table 4 are the average results from yield trials in five locations. Three of the subclones were lower in yield than the parent clone, and one, Pindar 70-31, is about equal. In some trials it out-yielded Pindar. Pindar is adapted to poor soil which accounts for 60% of the sugarcane area in Fiji, but its use is

Table 3. Fiji disease reaction of sublones derived from callus of susceptible and resistant parent clones

Parent clone	Rating	Number of sub-clones	Subclones disease rating[a] 1	2	3	4	5	6	7	8	9	% acceptable for field planting (rating 1–4)
Pindar	8	104	0	9	14	7	9	19	7	37	2	29
Ragnar	2	69	39	16	6	4	4	0	0	0	0	92
Badila	3	35	32	1	1	1	0	0	0	0	0	100
LF 66-9601	8	62	0	3	11	19	15	12	2	0	0	53
LF 63-274	7	7	0	0	0	3	3	1	0	0	0	42
LF 60-3879	8	28	0	8	4	4	7	4	1	0	0	57
Yasawa	2	6	6	0	0	0	0	0	0	0	0	100

[a] Rated on a scale of 1–9; 1 being resistant, 9 being highly susceptible.

Table 4. Average results from yield trials in 5 locations of Pindar and four of its subclones

Clone	Sucrose as % of fresh weight	Tons cane per hectare	Tons sugar per hectare	Disease rating[a] Fiji	Downy mildew
Pindar	14.5	81.6	11.8	8	4
Pindar 70-5	14.4	63.2	9.1	3	1
70-6	16.2	51.6	8.3	3	2
70-7	16.8	63.5	10.6	3	1
70-31	14.7	79.4	11.7	2	1

[a] Rated on a scale of 1–9; 1 being resistant, 9 susceptible.

limited because of susceptibility to Fiji disease. By virtue of its resistance to Fiji disease, subclone Pindar 70-31 has the potential of becoming a commercial clone of importance in Fiji.

The Pindar subclones are the fastest germinating clones in Fiji. They emerge within seven days after planting, while Pindar normally takes 10 to 12 days. Other characteristics are similar to the parent clone, Pindar.

4.1.3 Downy Mildew

Downy mildew disease of sugarcane is a systemic disease caused by the fungus *Selerospora sacchari*. When plants are infected, the organism grows out of the leaves through the stomata and sporulates. Plants can be infected at any age. Reddi and Gluinadi (1970) reported on methods of screening for resistance to downy mildew.

Table 5 summarizes the results of screening subclones for their reaction to downy mildew. As with eyespot and Fiji disease, some variants more resistant than the parent clone were obtained. Subclones of the intermediately susceptible clone LF60-3879 were highly resistant with a range in reaction from 1 to 0.

Table 5. Downy mildew disease reaction of subclones derived from callus of susceptible parent clones

Parent clone		Number of subclones	Subclone disease rating[a]							
Name	Rating		1	2	3	4	5	6	7	8–9
Pindar	4	4	4	0	0	0	0	0	0	0
Waya	6	4	0	1	1	0	1	1	0	0
LF 51-124	6	4	0	1	2	1	0	0	0	0
LF 60-3879	5	22	8	2	5	2	3	1	1	0

[a] Rated on a scale of 1–9; 1 being resistant, 9 highly susceptible.

Subclone Pindar 70-31, which has high resistance to Fiji disease, also has high resistance to downy mildew. The parent clone Pindar is intermediate in reaction to the disease.

4.2 Induced Variation by the Use of Mutagens

We attempted to increase the mutation frequency for given characteristics through the use of ionizing radiation (Heinz, 1973) and chemical mutagens (Mee *et al.*, 1969). We have no evidence of a higher mutation frequency for our selected characteristics using either method.

A clone X treatment effect was observed (Heinz, 1973 b). Two clones were killed and two clones survived when treated with either 2, 5, or 8 K/rads of gamma irradiation. We (Heinz and Mee, unpublished data) observed a clone X chemical interaction. Some chemicals, especially the mutagens methyl methanesulphonate (50 mg/l) and ethyl methanesulphonate (50 mg/l), are lethal to certain clones and nonlethal to others. The use of p-fluorophenylalanine (92 mg/l) in unsuccessful attempts to select haploid cells also shows differential toxicity among clones. (Maretzki, unpublished data).

These clone X mutagen treatment interactions indicate that more information is required before the optimum mutagenic treatment of sugarcane cultures can be determined.

5. Selection

The most effective crop-improvement use of suspension or callus culture techniques will occur when effective selection techniques can be coupled with directed genetic changes. We have been successful in screening for resistance to three diseases in subclones derived from cell cultures. However, selection for resistance to smut disease (*Ustilago scitaminea*) has failed.

The ideal screening tool appears to be the use of specific toxins or derivative media for selection of surviving cells. In this case resistance is probably based on

the presence or absence of a particular protein or enzyme. Undoubtedly there are a number of characteristics which could be identified for selection at the cellular level. Ultimately, however, identified variants will need to be tested for yield potential, including such characteristics as sucrose content, fiber, ash, starch, and acceptability for milling.

Selection at the cellular level should provide a basis for improvement of specific high-yielding clones, deficient in one or two characteristics. This becomes especially important if no replacement clones are available when, for example, a new pest or disease is introduced. Once the desired variant is identified, the process of selection for other desirable characters may remain as difficult as selection in sexually derived populations.

6. Conclusions

Cell and culture techniques in sugarcane have taken two major thrusts. One of them is related to modifications in the plant's genome due to passage through the tissue culture system. The other is concerned with the use of cultures for the elucidation of biochemical parameters. The former has a more immediate spectacular impact on agriculture as evidenced by selection for disease resistance which is probably due to chromosomal mosaicism. However, the latter provides the supporting framework. Future plans for use of cell culture techniques on sugarcane are predicated on the assumption that sugarcane can be genetically modified to program an ideal system in terms of sugar storage, disease resistance, and adaptability to physical environment. Sugarcane metabolism is central to the issue, and cell culture techniques provide a hopeful tool to increase our knowledge about the metabolic network. With cell cultures we find our tasks both increased and decreased. On the one hand, removal of normal constraints of plant growth may introduce complications such as increased genetic variants. On the other hand, some of the dynamic aspects of growth that are normally masked become accessible to experimental manipulation. Another cell culture advantage is that the very size of mature sugarcane imposes limitations on experimentation with the entire plant.

Commercial sugarcane is heterozygous, with germplasm from three to five *Saccharum* species. High-yielding disease- and pest-resistant clones have been developed during the past 65 years for most major sugarcane-producing areas. This was accomplished through conventional breeding and selection programs with 10 to 15 years required to identify and release a new clone. Once released, the clone can be vegetatively propagated as long as desired. Variation in most observed characteristics is available in the germplasm pool, with the frequency being low for some, i.e. Fiji resistance, and high for others, i.e. eyespot disease. By proper handling of the germplasm pool through breeding, the frequency of desired characteristics can be increased rapidly. No particular advantage is derived from propagation by tissue culture unless pressure for selection can be directed toward one particular characteristic such as has been done in the case of eyespot,

Fiji, and downy mildew diseases. The problems arising from the identification of disease- and pest-resistant clones that are also high-yielding are similar, whether selecting from subclones derived from callus or from clones obtained by conventional breeding techniques.

On the basis of our present knowledge, we believe that the greatest gains in the use of tissue culture will be realized when directed genetic changes can be coupled with effective cellular selection methods. The technical problems in developing these procedures are equally as great for sugarcane as they are for other agricultural crops, but the potential payoff warrants the effort.

References see page 207.

2. Propagation of Ornamentals by Tissue Culture

D. P. Holdgate

1. Introduction

Plant tissue cultures can be used for the mass propagation of plants; it may be argued that such a method of production could be a process to revolutionise some aspects of horticulture and agriculture. The production of millions of plants via suspension cultures and embryogenesis can be achieved. It is, however, impractical to handle such numbers in a commercially acceptable manner. For example, it is not feasible to transfer by hand or machine millions of small rooted carrot plants to the field. In most cases of embryogenesis an economic method for commercial utilisation needs to be formulated.

During the last five years, the in vitro propagation of genera from many families has been studied, perhaps in part stimulated by the commercial success of the tissue culture (meristem) propagation of the orchids. This apparently successful application hid for a period of time the serious shortcomings of tissue culture propagation when the techniques are not completely understood and controlled; many orchid plants produced by both small and large concerns contained virus, usually Cymbidium mosaic virus or the orchid strain of tobacco mosaic virus. The assumption made by many commercial propagators that the meristem method would automatically remove virus was, and is, all too frequently proved wrong, occasionally with disastrous commercial results for the grower of cut flowers. There is a tendency to refer to the "meristem" culture of orchids for propagation and forget the application of stem-tip culture in the production of disease-free plants. Stem-tip culture has been described by Hollings (1965, 1969, 1971) for producing virus-indexed *Dianthus* and *Chrysanthemum*. Several commercial nurseries have incorporated this relatively simple technique into their routine methods of propagation.

The total monetary value and cultivated area of these genera on a worldwide basis is not accurately known but they are of major importance as the statistics (Table 1 and 2) from the USA indicate. These levels have only been achieved by the use and maintenance of clean stocks derived from stem-tip culture.

Growers of other ornamentals are becoming more aware of the potential increase in yields and the quality which disease-indexed stocks could give. The basic economics and slowness in multiplying and bulking-up stocks from a single plant has possibly delayed progress at this commercial level, but tissue culture can now help in many cases to overcome these difficulties.

Unfortunately, variability in some tissue culture-derived plants has been used as a generality to label tissue culture automatically as a tool which will always result in genetic variation. This laboratory has produced hundreds of thousands

Table 1. US Flower seeds and Bulbs ranked by value of sale 1970

	Rank	US dollars
Flower seeds, total		1654130
Zinnia	1	305431
Marigold	2	183391
Sweet Pea	3	157065
Stock	4	98677
Petunia	5	85923
Aster	6	37574
Snapdragon	7	26954
All other		750115
Bulbs, total		10375341
Gladiolus	1	2984113
Lily, Easter	2	1642215
Iris, bulbous	3	1013511
Iris, rhizomatous	4	688243
Lily, other than Easter	5	644230
Daffodil	6	613336
Calladium	7	570928
Peony	8	350381
Dahlia	9	313201
Tulip	10	271703
All other		1283480

Table 2. US Rank of florist crops by total sales value

	US dollars		US dollars
1. *Chrisanthemum*	119630995	12. Tulip	4831395
2. Rose	60800568	13. *Hydrangea*	4274029
3. Carnation	55512579	14. *Begonia*	3409501
4. Cultivated foliage	50817378	15. Snapdragon	3205634
5. Geranium	24795246	16. Marigold	3201258
6. *Gladiolus*	20918107	17. Pansy	2688148
7. *Poinsettia*	20483032	18. *Gardenia*	2389191
8. *Azalea*	19544640	19. *Saintpaulia*	2310454
9. Orchid	12773754	20. Daisy	2241077
10. *Lily*	10541011	21. *Salvia*	2211391
11. *Petunia*	10345699	22. Hyacinth	2058626

of orchids in several genera, including *Cattleya*, *Cymbidium*, *Odontoglossum*, *Phalaenopsis*, and *Vanda* but observed mutations have been rare and limited to the occasional variegated leaf, and in one case a tetraploid plant produced from a diploid miniature *Cymbidium*. Several other orchid growers have had the same experience. Variations in other crop plants tested have, in our experience, been few or related to specific treatments, either prior to or during culture. Donor plants produced by or treated with irradiation or other mutagenic techniques which cause chromosomal aberrations are more likely to produce variable pro-

geny via tissue culture. Equally, variations are likely when cultures are treated with high levels of auxins or compounds which tend to upset cell division cycles or interfere with nucleic acid or protein synthesis. Should mutation or uncontrollable increases in ploidy occur, then the tissue culture system employed must be deemed unsuitable for that cultivar, but should not condemn all tissue culture systems.

There is substantial economic potential for the nurseryman, virologist and tissue culture scientist to work in close association to make desirable improvements in horticultural crops. There are, however, crops of national socio-economic importance which cannot be commercially viable for the tissue culture laboratory, e.g. *Narcissus*, and in such cases the problems may best be tackled by Government either at existing financed centers, or perhaps more suitably by commissioning work via an established commercial laboratory specialising in the use of tissue culture.

The present production techniques are labour-intensive, and quality-control for testing and maintaining disease-free stocks are costly. The demand for plants is insufficient for the creation of even a small number of commercially viable production units, at least until new techniques are available for handling larger quantities of plants. Immediate applications are centred around three main areas: (1) To assist the breeder by provision of stud-plants. (2) The rapid propagation of new cultivars. (3) To create and maintain stocks of disease-indexed plants of selected clones from existing cultivars in sufficient quantities to be of practical value to the commercial grower.

2. Assistance to Breeder

2.1 Introduction of New Varieties

To produce a new cultivar requires many years of patient cross-fertilisation and selection trials. Rarely can any programme produce more than a small number of new cultivars and frequently only one and sometimes none. Thus, those plants selected must be sold at a price and in sufficient quantity to cover not only the cost of the trials, but also the cost of multiplying sufficient stocks of the selected plants. In cases requiring vegetative propagation, the selection and build-up to the point of sale may take 7 to 15 years. During this period there is a substantial risk of an unacceptable infection, such as virus in *Freesia* and *Lily* when the entire potential and substantial financial investment would be lost.

In the case of *Freesia*, for example, (personal communication, Parigo Horticultural Co. Ltd., Bourne Road, Spalding, Lincs.) a breeder's first sales may be eight years after the final selection trials and represents an investment programme of at least 10–11 years. By using tissue culture, this long and expensive period of stock build-up could be reduced, and allow the breeder to introduce the new variety within 2–3 years from the final selection trials. The value of tissue culture is, therefore, in the reduction of the investment in conventional methods of propa-

gation and a reduced risk of viral infection, plus the value of the glasshouse space freed by an earlier distribution of the new variety, less the cost of the tissue culture plants. In cases where breeders' rights and issuing of propagating licences are agreed, an income to the breeder can be realised earlier. A conservative estimate would be that three new varieties could be introduced using tissue culture where one introduction is achieved with present methods. A greater use of tissue culture with coordination of facilities could increase this ratio.

The marketing of new disease-indexed varieties earlier should be of significant value to the primary commercial growers. The healthy plants should be highly productive and the saleable produce should be well above average quality with increased market value. New varieties almost invariably command higher prices because of the scarcity and novelty value and for the improvements which the breeder will have provided, e.g. colour, vase life, length of stem, resistance to flower spotting and the freedom from deformities which frequently occur in flowers from virus-infected plants.

The comments above can apply to many ornamental breeding programmes and the application of tissue culture in the case of *Begonia*, *Freesia*, *Lily*, and *Saintpaulia* could make available new introductions 5 to 6 years earlier, and also would provide selected clones of *Anthurium* which could not otherwise be marketed.

2.2 Provision of New Genotypes

The breeder's genetic pool is frequently extremely large and the variations possible are enormous, but the combination of genes in the desired manner by repeated backcrossing, may take an excessively long time. The application of pollen culture and the production of haploid plants has obvious potential benefits (see Chap. II.1 of this Vol.). Specific desirable gene combinations may be linked in a haploid form and then converted to the fertile homozygous diploid or polyploid forms which are usable in breeding programmes. However, this type of service must be considered a long term programme as: (1) Diploids derived from haploids will show variation and are unlikely to be of substantial value as new varieties in their own right. (2) Substantial screening and careful selection will be necessary to segregate those plants containing the important genes whether singly or in desired combinations. (3) Further desirable combinations will only be obtained by repeated crossing, selection and further pollen or anther culture and identification of the new genetic combinations obtained.

The breeder who wishes to make the greatest use of the production of such homozygous plants will need to maintain a substantial stock of well-documented genotypes, as the technique cannot be considered a short cut or an inexpensive source of "instant" new varieties.

The creation of polyploids as new cultivars from existing lines by treatment with colchicine is valuable in some cases, but the selections must be carefully controlled because there is a danger that even slight chromosomal variations may occur, followed during subsequent tissue culture propagation by a progressive build-up of aberrant genotypes. Whenever feasible, plants produced by the col-

chicine treatment should be checked carefully for chromosome number and composition before multiplication.

Further genetic variations can be obtained by culturing plants treated with various mutagenic agents. Rapid multiplication of the culture from such plants which may have abnormal and unstable chromosome complements, and subsequent organogenesis can result in a wide range of phenotypes.

These variations may show up progressively during conventional methods of propagation, or possibly several years later when the plants are under another grower's control.

It has been our experience that plants of *Saintpaulia* produced via callus derived from donors, which are mutants caused by irradiation, demonstrate a substantial variation in flower for and habit. Although no cytological analysis was made to ascertain chromosomal changes, plants produced from donors of more normal origin proved uniform in character.

Although searching for new stable genotypes in this manner may be initially expensive, it is a practical method for increasing the chance of realising the full potential and financial benefit from new cultivars.

3. Creation and Production of Stocks of Disease-index Plants of Selected Clones

Many commercial crop plants, particularly those propagated by vegetative means, contain systemic bacteria, fungi and viruses which affect the yield and frequently the quality and appearance of the saleable item. It is economically desirable to produce plants which are free of such debilitating organisms. In most cases the grower who produces the best quality commands the best price at the market level, and it follows that a producer of quality goods has a greater profit potential.

In many countries *Chrysanthemum* and *Dianthus* plants derived from virus-indexed parent stocks are readily available. There is however effectively no commercial source of virus-free plants of other ornamental crops for the average grower. Some of those plants which have been made virus-free are difficult to propagate. It will be many years before sufficient numbers are available to provide samples for the general grower, e.g. *Narcissus tazetta* cv. Grand Soleil d'Or has been made virus-free (HOLLINGS, 1969, 1974; STONE, 1973) but the conventional methods of propagation are relatively slow and therefore many years are required to create a sufficiently large number of bulbs for planting to be (1) commercially viable, and (2) to have a reasonable expectation of retaining the virus-indexed state of the stock for a substantial period of time. A full discussion on this topic is presented by QUAK (1957, see also Chap. V.2.3 of this Vol.)

Commercial quantities of virus-indexed freesia, gladiolus, hyacinth and lilies could be made available within two years by employing tissue culture techniques for the removal of virus (BRANTS and VERMEULEN, 1965; ALLEN, 1974; ASJES *et al.*, 1974) and propagation. The effectiveness of any scheme to produce disease-

free plants is related to the efficiency of the testing procedures used, but all require large capital investment. There are effectively 3 methods available: (1) the use of serology which can give very rapid results for specific viruses, but is limited to a ready source of antisera for the specific viruses under test, (2) the use of indicator plants which, when subjected to even small levels of virus infection, would generally show characteristic local lesions. This technique, however, has limitations in the source of suitably responsive indicator plants and a relatively large area of suitably isolated glasshouses if large numbers of routine tests are to be carried out, and (3) the use of the electron microscope of sufficient capacity to detect both rod and isometric viruses. The detection of virus using the electron microscope can be extremely rapid by a "quick-dip" technique, however, it must be realised that in attempts to produce a virus-free plant, the operator is always dependent on a negative test result and the operator's technique must, therefore, be beyond reproach. It is the practice in Twyford Laboratories that repeated assays are made using the electron microscope, and where possible these are backed up with tests using suitable indicator plants and/or serological techniques; further repeated tests are made during the build-up of stocks to ensure that the virus freedom was not due merely to a low concentration of virus in the initial test material.

Having established that the parent plant is virus-free, a wide range of tissue, for example from older petioles, leaf lamina and stem sections, is available for tissue culture. There may be a range of theoretical and factual arguments for not extending the initial inoculum to these more mature tissues, but the final decision must be related to the experimental findings relating to the uniformity of cultures and the plants produced.

4. Crop Evaluation for Tissue Culture

Accurate statistics for the horticultural trade cannot be obtained, but those published by the International Association of Horticultural Producers, and the US Department of Commerce, Census of Agriculture, give good indication of size and value. Areas used for cut flowers cannot be assessed accurately because of double cropping, but in Western Europe cut flower crops occupied between 14 and 15 thousand acres of glasshouse space in 1971 and a further 30 thousand acres outdoors. France, Italy, Holland and West Germany account for 85% of this total. Production in recent years has been increasing in all EEC countries except Britain and Denmark. West Germany is now the largest producer of ornamentals in Europe with a production value up by 151% in the years 1957 to 1968; despite this, Germany is still the largest importer of cut flowers in Europe.

Traditionally, Holland has acted as a primary distributor for the resale of flowers to other countries, particularly Germany, Switzerland and Scandinavia, but also North America. The clock auction system tends to act as a quantity and price regulator for the entire European trade, even though many growers with increasing production are exporting direct from their country to specific markets throughout Europe. The sales profile of flowers and pot plants can be explained in

part by such factors as population movements and the degree of urbanisation, but more especially by the increase in buying power of individuals in most of the EEC countries which, e.g. Belgium, has been 6–7% per annum. In most societies flowers and pot plants are considered to be nonessential and consumers will only purchase regularly when incomes are such that individuals have free disposable cash.

Attempts to discover which plants are the most interesting objects of study from the economic point of view may not always be obtained from national and international horticultural statistics. (e.g. the Census of Agriculture 1969, Volume V, Special Reports Part 10, Department of Commerce, United States Bureau of the Census; and the International Association of Horticultural Producers, European Horticultural Statistics published yearly by The General Secretariat, I.A.H.P., Stadhoudersplantsoen 12–18, Postbox 361, Gravenhage, Netherlands). These may lag so far behind present trends in the economic, scientific and commercial developments, as to be of only limited value. Analysis indicates that a sound commercial application of tissue culture to produce large numbers of disease-free plants of existing varieties cannot be made for those plants which are traditionally of national importance, such as bulbs in Holland and England, and produced in such volume that the individual price is very low. However, from a long-term national point of view, and in the case of many specialist breeders and growers, the investment in disease-free plants and their rapid propagation by tissue culture, may be justifiable. It is, therefore, necessary, not only to consider general trends in national and international trade, but also the social and economic effect of a genera becoming nonviable as a result of mass infections and consequently poor quality. With ease of intercontinental distribution, plants become challenged by an increasing variety of pathogens and where such traditional, and in bulk terms valuable crops, are so threatened, a national scheme combining the expertise of both Government and commercial establishments, to produce and maintain disease-free stock could be essential in the future, and from a grower's point of view commercially desirable now.

Certainly the provision of the enormous number of bulbs necessary to raise and maintain the health level of many bulbous crops could be beyond the capabilities of existing flower producers and propagators, as conventional procedures cannot scratch the surface of producing disease-indexed stocks. The current economic value of *Narcissus* in the UK is significant with production approaching 100 million blooms and valued at well over £600000 (CLARKE, 1974). Dutch production is shown in Table 3.

Using tissue culture as a rapid method to create an initial stock of *Narcissus* and other crops would provide the foundation for the creation of healthy stocks of selected clones in a more commercially acceptable time.

The potential of tissue culture for some of the newer crops is better assessed from grower requirements than official statistics. In the case of *Anthurium* (see Table 4) acreage has increased dramatically; plants are derived from seed with substantial variation in the most important aspects of colour, quality, annual yield and time of first flowering. There is a strong demand for vegetatively produced stocks which conventional means cannot satisfy. The application of tissue culture will enable the breeder to provide the cut flower *Anthurium* grower with

Table 3. Dutch exports 1973

	Cut flowers	Pot plants
	(million guilders)	
W. Germany	546.5	40.8
France	26.7	6.0
Benelux	11.6	5.0
Italy	9.3	5.9
U.K.	4.4	3.6
Ireland	0.7	0.1
Denmark	0.1	1.7
Total EEC	599.4	64.0
Other European countries	59.6	19.2
Countries outside Europe	4.3	2.4
Total exports in 1973	663.3	85.6

Area (hectares) used for cultivation of various bulbous and tuberous plants in Holland

	1966	1972
Hyacinth	820	787
Tulip	5917	5767
Narcissus	1395	1551
Gladiolus	1732	2103
Miscellaneous	1669	2295
	11533	12503

Total export of all bulbs, 1972

In:	
millions of kg	112.60
millions of guilders	432.95

the clonal selections necessary to improve yields and quality and combat the ever-increasing labour, fuel and material costs. Tissue culture-derived plants should prove to be of a very high standard and raise the general quality of produce for sale, and, providing the costs are realistic, should be beneficial to the good growers, but will not compensate for poor growing technique.

Consumers are progressively demanding good value for money as growers are becoming more conscious of the necessity to produce high-quality items which fetch better prices, are sold faster and can produce a greater return on capital employed. Tissue culture will only be a valuable tool in both the creation of the healthy stock and the initial multiplication if the size and form of the plants for delivery to the grower are acceptable and at the right price. The grower relates costs more to existing material than to the potential increase in income from as yet unrealised higher yields, better quality and lower prices. Only occasionally

Table 4. *Anthurium andreanum*

	Total No. flowers		Price per flower (guilders)	
	Year 1974	Year 1973	Year 1974	Year 1973
January	109001	67903	1.7511	1.7529
February	89206	52717	1.9372	2.1071
March	84582	44553	2.02	2.19
April	112096	48224	1.88	2.13
October	300551	188680	1.21	1.32

Growth of area of *Anthurium andreanum* in cultivation in Holland

Year	Acres	Percentage growth
1972	99	—
1973	124	25
1974	168	36
1975[a]	200	19

[a] Anticipated figures.

can plants be produced economically in tissue culture for direct production planting, e.g. *Orchids*, *Anthuriums* and nonvariegated bromeliads. In most cases growers believe that the cost of tissue culture-derived plants is too high and that they are more suitable as foundation or mother stocks. At present many growers desire to utilise tissue culture, and although the application of such a new development presents some worries about survival rates and eventual product uniformity, a combination of scientific know-how and the grower's ability to adapt his husbandry techniques should overcome cultural problems. There is more than sufficient evidence to demonstrate that plantlets from tissue culture can exhibit a degree of uniformity acceptable to the commercial grower, although it may prove necessary to vary the tissue culture media according to genera, species, variety and even clone to provide uniform planting material. Close cooperation between grower and scientist is essential at this stage of development to ensure that trials are performed on a sufficiently large scale and in a suitable manner. The measure of success in such trials should be the acceptability of the product by the customer and the contribution to the commercial enterprise as measured by comparison of overall costs and efficiency, rather than a precise growth parameter such as leaf size.

The uniformity of growth of tissue culture-derived plants is suitable for incorporation into the fully integrated, computerised monitoring programme for adequate management and control of delivery, growing and marketing functions recently introduced (CROSSLEY *et al.*, 1973). This is utilised and extended by ROCHFORD (personal communication) with considerable commercial benefit, enabling significant increases in production and reduction in costs.

The most acceptable application for growers wishing to use existing cultivars will be in the production of disease-indexed, high-quality stock plants (e.g. *Pelargonium*, *Begonia*, *Narcissus*, and *Nerine*) to be used as the mother-stock material for conventional propagation methods. The application of systems involving the production of hundreds of thousands of plantlets, which may reduce costs via embryogenesis in liquid culture, may be warranted only at a later date when systems suitable for handling and planting have been developed.

Earlier commercial benefits are possible for the breeder by using tissue culture for the propagation of new varieties. Assistance in the provision of new genotypes can be undertaken, although in the case of pollen and anther culture, substantial research is required before general application can be anticipated. However, immediate advantage can be taken by reproducing a sufficient number of scarce parental lines for subsequent F_1 seed production; thus creating genotypes in quantities which would otherwise be commercially nonviable.

5. The Establishment of Cultures

5.1 Selection and Preparation of Tissue

It is now well-proven that the production of callus from various plant sources is not difficult (see Chap. I.10 of this Vol.). The majority of plants studied, however, have been dicotyledonous and the work on monocotyledons has for some time been considered by many as more difficult, but there is ample evidence that callus from monocotyledons can be produced without difficulty (STRAUS, 1954; CARTER *et al.*, 1967; WILMAR and HELLENDOORN, 1968; DAVIES and HELSOP, 1972; HUSSEY and WYVILL, 1972; BAJAJ and PIERIK, 1974; HOLDGATE *et al.*, 1975). The concentration of research on callus from the herbaceous dicotyledonous plants may frequently be because of their greater convenience as laboratory specimen material, and not for any particular applied or commercial value.

The induction of callus may occur by using tissue from any part of the plant, but the origin of the inoculum is generally more dependent on the aims of the experimenter than of any difficulty in culturing. In the case of plants used for propagation purposes, root tissue is rarely used because of contamination problems, and because of the substantial disadvantage of constituent cells being of varying ploidy and resulting in uneven growth (TORREY, 1961, 1965, 1967, 1971). The problem of ploidy may also apply to more mature tissue from aerial parts of the plants (BENNICI *et al.*, 1971) and care must be taken to select tissue of known composition or proven performance. It is the aim of the plant propagator to produce, to the specifications of a grower or breeder, large numbers of genetically uniform plants—not a mass of cells more suitable for nutritional, biochemical or cytological studies. It is therefore advisable to utilise, whenever possible, young tissue consisting largely of immature cells, most likely free of polyploid cells, as the initial inoculum. Such a tissue is most frequently found in the minute scale leaves, leaf primordia and the meristematic tissue of both dormant and actively growing buds. However, inocula derived from developing floral tissue may also be conve-

niently and successfully used as in the case of *Gladiolus* (ZIV *et al.*, 1970; DAVIES, 1971), *Freesia* (BAJAJ and PIERIK, 1974; HOLDGATE *et al.*, 1975), *Phalaenopsis*, *Saintpaulia* (HOLDGATE *et al.*, 1975) and *Chrysanthemum morifolium* (BUSH *et al.*, 1974) and in the case of *Gerbera* (PIERIK *et al.*, 1973; MURASHIGE, 1974) and *Chrysanthemum cinerariaefolium* (ROEST and BOKELMAN, 1973), the capitulum.

6. Preparation of Tissues and Media

Adequate preparation and surface sterilization of plant tissue is essential for successful aseptic technique. Clonal plants used in commercial laboratories are frequently of high value and in short supply, and there is often no room for experimentation with sterilization technique. An accurate assessment of the extent of the surface microflora, coupled with past experience of sterilizing similar tissue, is desirable before deciding on sterilization. In general terms the following sequence has been successful when tissue is to be isolated from buds: (1) Remove soil, dead and diseased tissue. (2) Dip in 70% Ethanol. (3) Using commercial bleach as principal sterilant immerse for 3 to 15 min in a 10% (v/v) dilution. (4) Wash in sterile water, first a quick wash and then twice for five minutes. (5) When further short-duration sterilization is necessary during dissection it should be followed by copious washing during dissection.

6.1 The Nutrient Media

6.1.1 Type

The choice of liquid or solid media may depend on the source of the explant and the objective of the culture. Many workers, particularly those growing shoot tips, prefer to use a liquid media with the inocula supported on a filter bridge (HØLLINGS and STONE, 1968) above the media. In Twyford Laboratories, solid media has been found convenient, cheaper and it produces consistent results. Only on rare occasions has it been justified to employ expensive labour to make filter bridges or to purchase alternatives.

6.1.2 Composition

Extremely complex media are usually unnecessary for general plant propagation. Variations of the media developed by KNUDSON (1922), MURASHIGE and SKOOG (1962), or by WHITE (1963) are generally effective at both macro and micronutrient levels with sucrose as the carbon source. The addition of plant extracts such as banana fruit, coconut milk and aqueous leaf extracts (used particularly in orchid culture) have no great advantage, and are too expensive for use in Europe. Carefully regulated additions of growth-promoting substances can be made to the media. The presence of cytokinins at concentrations ranging from 0.5 to 10 mg/l, depending on the tissue and chemical employed, have been found most effective in

producing the type of growth most suited for plant propagation. However, MURASHIGE (1974) indicates that 30 mg/l of kinetin is required in *Gloxinia.* IAA is used when an auxin is required at the lowest effective concentration which can only be decided by experimentation. NAA is rarely used and 2,4-D is never used in the author's laboratory for routine cultures. There is adequate data presented which shows that 2,4-D can cause a rapid increase in ploidy in cultures derived from a range of plant tissues, (WILMAR and HELLENDOORN, 1968; SUNDERLAND, 1973) to the exclusion of the original chromosome complement.

Although the ratio of cytokinin to auxin is important during the process of organogenesis (see SKOOG and MILLER, 1957; REINERT, 1973; FONNESBECH, 1974 a) it does not follow that exogenous auxin is obligatory. In preparing a media to use in tissue culture for plant propagation, it must be realised that most published modifications are related to the production of callus for studying nutritional, physiological, biochemical and cytological processes. It may frequently be a disadvantage to produce conditions which create "optimal" callus growth rates which may reflect such rapid rates of cell division that an incomplete replication of the genome or some other serious chromosomal aberration may occur. Although relatively simple media can be employed for propagation purposes, it is imperative that no essential nutrient, inorganic or organic, is in fact lacking. Substances which are likely to cause variations at the nucleic acid levels should be omitted. It is possible that variations observed in plants produced by tissue culture have been produced in nutrient systems which result in the most rapid rates of cell division or contain 2,4-D (WILMAR and HELLENDOORN, 1968; NISHI *et al.*, 1968).

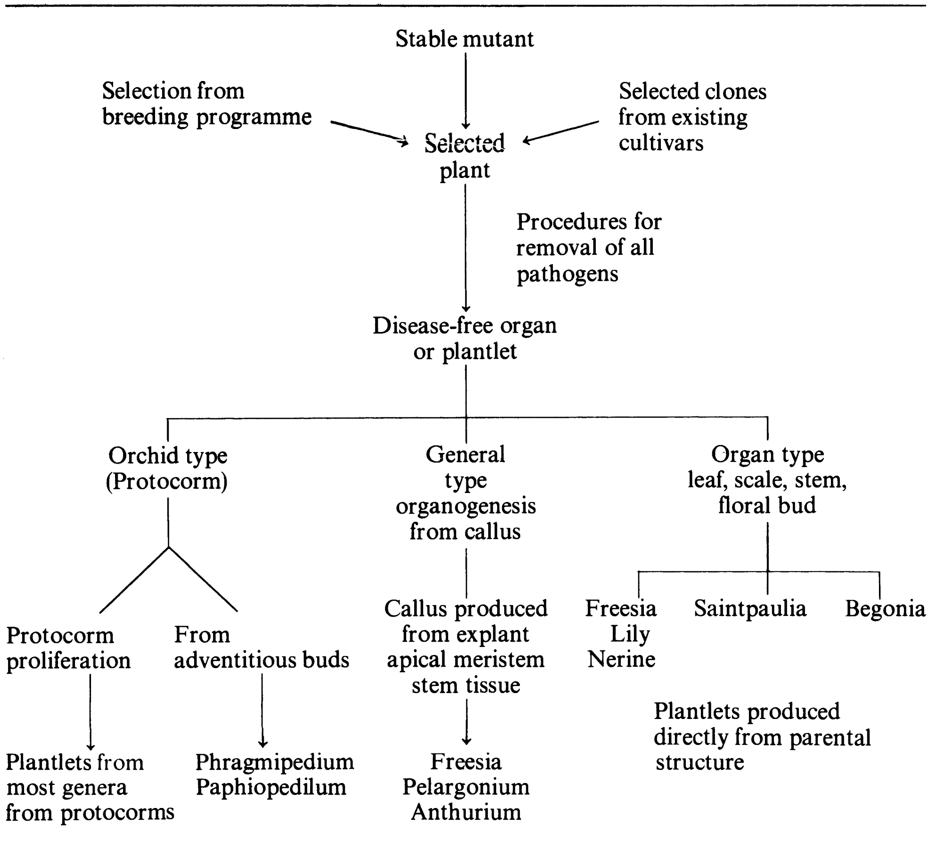
Although regular and repeatable regeneration of an increasing range of plants is possible, a complete understanding of the nutritional and hormonal balance is lacking. The frequent increase in ploidy with age of the cultures [TORREY, 1967; BENNICI *et al.*, 1971 (see Chap. III.1 of this Vol.)] strongly suggests the use of young cultures for propagation purposes when uniform flowering of regenerated plants can be obtained (DAVIES and HELSOP, 1972; BEN-JAACOV and LANGHANS, 1972).

In a recent review, MURASHIGE (1974) states that 3 stages may be considered when using tissue culture for propagation. The assumption that a disease-free plant is used (or automatically available or created during culture) has caused serious setbacks to numerous orchid growers and, therefore, from a commercial horticultural point of view, a fourth stage must be included by any laboratory offering tissue culture and propagation services. The initial stage to be added is the pathological function of identifying and subsequently removing any disease present to the specifications of the customer prior to entering stage 1 as given by MURASHIGE. Thus, Table 5 is a scheme demonstrating the sources and stages which operate in Twyford Laboratories Ltd. in employing tissue culture to propagate selected plants for the ornamental industry.

6.1.3 Initiation of Cultures for Production Purposes

Plants received for propagation are examined and tested for infections of fungi, bacteria and virus. Lack of symptoms in a plant does not necessarily mean a low

Table 5. Scheme for propagation of plants by tissue culture as at present used at Twyford Laboratories Ltd.



level of virus infection but may indicate a certain resistance to an infecting pathogen. Although it is basically assumed that fungal and bacterial colonies are present, it is useful to know what these are. Plants, which by all reasonable tests are free from virus, are cultured in a manner which ensures the removal of the other contaminating organisms. Once all surface contaminants have been removed and the tissue washed several times with sterile water, selection of tissue which is free from systemic contaminants is made for initial culture. The tissue generally used is connected in some way to the terminal or axillary buds. The inoculum will consist of an apical dome plus some leaf primordia, or small leaf segments. Unless bacterial spores have entered the bud during normal watering of the donor plant in the greenhouse, they are generally free of bacteria and fungi and no contamination problems should be experienced when establishing a suitable culture.

Plants, which are found or suspected of containing virus, are either rejected as unsuitable for propagation and returned to the breeder or grower for destruction, or if the plant is considered of sufficient merit, attempts will be made to produce a virus-free stock before mass propagation is started.

The merits of the various treatments of plants and tissues for removal of virus, will not be dealt with in any detail here, the work having been reviewed in detail by Quak (see Chap. V.2 of this Vol.) but the following summarises the general approach adopted in this laboratory.

6.1.4 Distribution of the Virus

Obtaining virus-free plants is often dependent on certain peculiarities of virus distribution within the plant, e.g. cucumber mosaic virus rarely invades the bulb scales of lilies and by culturing pieces of tissue from such a region, there is a good chance of isolating many plants which are free of that virus (BRIERLEY, 1962). Similarly, several researchers (HIRTH and LEBEURIER, 1965; HANSEN and HILDEBRANDT, 1966; CHANDRA and HILDEBRANDT, 1967) have demonstrated that tobacco callus cells from tobacco mosaic virus (TMV)-infected plants consist of a mixture of infected and uninfected cells and that both TMV-infected and TMV-free plants, can be produced from such calli. Virus-symptomless plants of *Gladiolus* (SIMONSEN and HILDEBRANDT, 1971) and *Pelargonium* (ABO EL-NIL and HILDEBRANDT, 1971) have also been obtained via stem callus and anther cultures.

6.1.5 Meristem Tip Culture

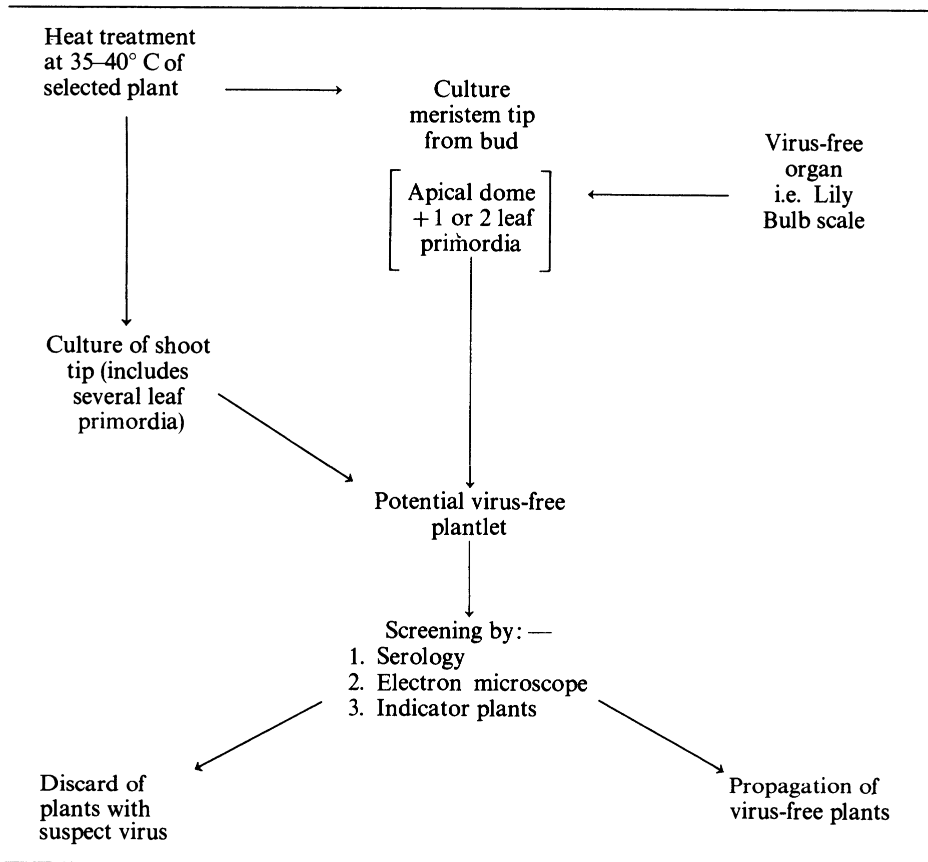
Where the virus is more generally distributed throughout the plant, successful elimination is more likely to be achieved with meristem tip cultures, e.g. cymbidium mosaic virus can be eliminated by meristem tip culture (MOREL, 1960). However, ample proof now exists to show that viruses are present in the meristem and although in many cases the virus is gradually lost during culture (HOLLINGS and STONE, 1964; WALKEY and WEBB, 1968) it cannot be guaranteed. LAWSON (personal communication) suggests that tobacco mosaic virus orchid strain, cannot be removed by meristem tip culture of *Cattleya*, irrespective of how small the inoculum. Even so, careful meristem cultures are always worth attempting for their speed and low costs.

Chemicals such as 2,4-D and thio-uracil which interfere with nucleic acid metabolism have been employed for eliminating virus (HOLLINGS, 1965). The use of such chemicals, however, is not advocated because the dose required cannot be properly judged and can equally attack host RNA and DNA with the probability of adversely affecting the genome.

6.1.6 Heat Treatment

Virus-free stocks are also obtained following heat treatment at 35–40° C for a period from two weeks to several months. Immediately following this treatment, small shoot tips or meristem tips are placed into culture and of those which survive some may be free of virus. Heat treatment may present disadvantages, as HOLLINGS (1965) has experienced variability in plants derived from axillary buds of the same parent plants; in the author's laboratory considerable clonal variation in *Pelargonium* in respect to heat tolerance has been observed. Plants which are chimera, or mutant having a tendency to chromosomal instability may well pro-

Table 6. Simple scheme employed for selecting plants for culture



duce significantly variable plants as a result of heat therapy. Behaviour of tissue from chimeras during meristem shoot tip culture is unpredictable. For example, culture of numerous variegated bromeliads invariably results in the production of the monochromic forms, green, white, pink and in numerous variations of the chimeral forms. Variation in cell division rates can be experienced in tissues of the same genetic origin once control of the organised structure is removed (TRAN THANH VAN *et al.*, 1974). Thus some basic variation may be created when chimeras are subjected to heat therapy and subsequent meristem tip cultures. Known chimeras and plants produced by mutagenic agents are not, therefore, subjected to such treatment on a commercial scale in Twyford Laboratories at the present time. Table 6 illustrates the routine for selecting disease-free plants.

6.1.7 Present Applications

Table 7 lists the plants of some ornamental significance which have been grown in vitro, but this does not necessarily mean that systems suitable for commercial production have been created. Many cultural systems described and functional at

Table 7. Ornamental plants with demonstrated potential for clonal multiplication through tissue culture

Family	Genus	Reference
1. Foliage		
Agavaceae	*Draceana*	MURASHIGE (1974)
Araceae	*Philendron* sp.	MURASHIGE (1974)
		HOLDGATE *et al.* (1975)
Begoniaceae	*Begonia Rex*	SHIGEMATSU and MATSUBARA (1972)
Blachnaceae	*Woodwardia fimbriata*	MURASHIGE (1974)
Cyatheaceae	*Alsophila australis*	MURASHIGE (1974)
Davilliaceae	*Nephrolepsis bossoniansis*	MURASHIGE (1974)
	Nephrolepsis exaltata	MURASHIGE (1974)
Marantalea	*Calathea*	MURASHIGE (1974)
Pterdiaceae	*Adiantum cuneatum*	MURASHIGE (1974)
	Microlepia strigosa	MURASHIGE (1974)
	Pteris argyrea	MURASHIGE (1974)
	Pteris cretica	BRISTOW (1962)
2. Foliage and/or flowers		
Amaryllidaceae	*Hippeastrum*	MII, *et al.* (1974)
	Ipheion	HUSSEY and WYVILL (1973)
	Amaryllis	MURASHIGE (1974)
		HOLDGATE *et al.* (1975)
	Narcissus	HOLDGATE *et al.* (1975)
		HUSSEY and WYVILL (1973)
	Alstroemeria	ZIV *et al.* (1973)
	Nerine	HOLDGATE *et al.* (1975)
	Clivia	HOLDGATE *et al.* (1975)
Araceae	*Anthurium andreanum*	PIERIK *et al.* (1974)
	Dieffenbachia	HOLDGATE *et al.* (1975)
	Caladium	HOLDGATE *et al.* (1975)
Begoniaceae	*Begonia* spp.	MURASHIGE (1974)
		FONNESBECH (1974)
		HOLDGATE *et al.* (1975)
		RINGE and NITSCH (1968)
Bromeliaceae	*Aechmea fasciata*	HOLDGATE *et al.* (1975)
		MURASHIGE (1974)
	Ananas comosus	HOLDGATE *et al.* (1975)
		MURASHIGE (1974)
	Cryptanthus bivittatus	HOLDGATE *et al.* (1975)
		MURASHIGEN (1974)
	Cryptbergia	HOLDGATE *et al.* (1975)
		MURASHIGE (1974)
	Dyckia sulphuria	MURASHIGE (1974)
	Bilbergia	MURASHIGE (1974)
	Nidularium	HOLDGATE *et al.* (1975)
	Neoregelia	HOLDGATE *et al.* (1975)
Euphorbiaceae	*Euphorbia pulcherrima*	LANGHE *et al.* (1974)
	Zalanchoe	MURASHIGE (1974)
Geraniaceae	*Pelargonium hortorum*	CHEN and GALSTON (1967)
		PILLAI and HILDEBRANDT(1968, 1969)
		ABO EL-NIL and HILDEBRANDT (1971)
		HOLDGATE *et al.* (1975)

Table 7 (continued)

Family	Genus	Reference
Gesneriaceae	*Gloxinia*	HARAMAKI and MURASHIGE (1972)
	Streptocarpus	APPELGREN and HEIDE (1972)
	Saintpaulia ionantha	HOLDGATE *et al.* (1975)
		KUKULCZANKA and SUSZYNSKA (1972)
Liliaceae	*Lilium speciosum*	ROBB (1957)
		HOLDGATE *et al.* (1975)
	Lilium longiflorum	HACKETT (1969a, b)
		HOLDGATE *et al.* (1975)
	Convallaria	SHERIDAN (1968)
		HOLDGATE *et al.* (1975)
	Hyacinthus	PIERIK and WOETS (1971)
		HOLDGATE *et al.* (1975)
	Tulipa	HUSSEY and WYVILL (1973)
	Scilla	HUSSEY and WYVILL (1973)
Primulaceae	*Cyclamen persicum*	STICHEL (1959)
Iridaceae	*Freesia*	BAJAJ and PIERIK (1974)
		DAVIES (1971), HOLDGATE *et al.* (1975)
	Gladiolus hortulans	ZIV *et al.* (1970)
	Sparaxis	HUSSEY and WYVILL (1973)
	Schizostylis	HUSSEY and WYVILL (1973)
Asteraceae	*Chrysanthemum merifolium*	HILL (1968)
		BEN-JAACOV and LANGHANS (1972)
	Chrysanthemum cinerariaefolium	ROEST and BOKELMANN (1973)
	Gerbera jamesonii	PIERIK *et al.* (1973)
		MURASHIGE (1974)
Caryophyllaceae	*Dianthus caryophyllus*	HACKETT and ANDERSON (1967)
	Phlox drummondi	KONAR and KONAR (1966)
Solanaeae	*Petunia inflata Hybrida*	RAO *et al.* (1973a)

3. Orchidaceae. The following is a list of orchid genera propagated at Twyford Laboratories. *Arachnis, Aranda, Aranthera, Ascocenda, Ascofinetia, Brassia, Calanthe, Cattleya, Cymbidium, Dendrobium, Dactylorchid, Doritaenopsis, Epidendrum, Lactia, Lycaste, Miltonia, Miltonidium, Neostylis, Neotti, Odontioda, Odontoglossum, Odontonia, Oncidium, Ophrys, Paphiopedilum, Phaius, Phalaenopsis, Phragmipedium, Pleione, Renanthera, Renanthopsis, Rhyncostylis, Sophronitis* and intergeneric crosses of the *Cattleya* tribe, *Vanda Vascostylis, Vuylstekeara* and *Zygopetulum.*

the test-tube level, are distinctly unsuitable for producing plants in the numbers, uniformity and at the price the grower is prepared to pay.

The use of meristem tip cultures for the routine production of virus-free *Chrysanthemum* and *Dianthus* stock plants is well known. The mass propagation by callus production and subsequent organogenesis has been described (HILL, 1968) and confirmed more recently (BEN-JAACOV and LANGHANS, 1972; EARLE and LANGHANS, 1974 b). However, attempts to modify and extend the previous work to be commercially viable, failed on cost of production. The laboratory plants were smaller than cuttings from mother stocks grown in warm climates such as Sardinia, Malta and the Canary Islands and would not fit into existing growing methods. The work of EARLE and LANGHANS (1974 b) does not result in a

sufficiently improved product to compete financially with existing conventional procedures using disease-free mother stock. However, extended work on *Chrysanthemum* by HILL and colleagues at Twyford Laboratories (unpublished results 1965–68) and by BUSH *et al.*, (1974), demonstrated the feasibility of using most parts of the adult flowering plant, including receptacle and tissue from the ligulate floret as the source of explants. The potential in rapid multiplication of a new mutant or a newly bred variety for earlier introduction to the market was demonstrated and localised mutations in florets may be utilised if cultured at an early stage. The chimeral nature of many cultivars of *Chrysanthemum* and other ornamentals, suggests that the technique may not be 100% successful, even though cultures derived from ligulate florets of the cultivars White Fred Shoesmith and Woking Scarlet, produced uniform plants, flowering true to type. BEN-JAACOV and LANGHANS (1972) found that plants regenerated during the early part of cultural cycles of callus derived from *Chrysanthemum* Indianapolis White c.v. Giant were true to type, but as the cultures aged, some 28% of the plants showed yellow flowers and some change in leaf habit.

The belief that tissue-culture propagation of plants can be of economic benefit and more generally applicable than at present, is widely held. Specific commerical parameters have not, and cannot, except in the case of orchids, be presented. The application is centred now on several crops, of which those of greatest importance, other than the orchids, are *Anthurium andreanum* and *Anthurium scherzerianum*, *Lily*, *Pelargonium*, various types of *Begonia*, *Freesia* and several crops produced by bulb, corm or tuber, and although the system for *Chrysanthemum* has not to the author's knowledge been used commercially, it is available for immediate use. It is not possible to assess the accurate present-day value of all these crops and what the total effect on existing standards would be if tissue-culture procedures could be commercially exploited by the growers. The interest of some leading breeders and growers suggest that significant desirable changes in variety, quality and plant health could be achieved, but tissue culture is an expensive process and its use may be excluded by its cost. We find it difficult to utilise tissue culture to propagate *Asparagus* because of the large capital expenditure required to replant significant areas with selected clonal material. A similar situation with some flower crops could be overcome by repeated use over a number of years.

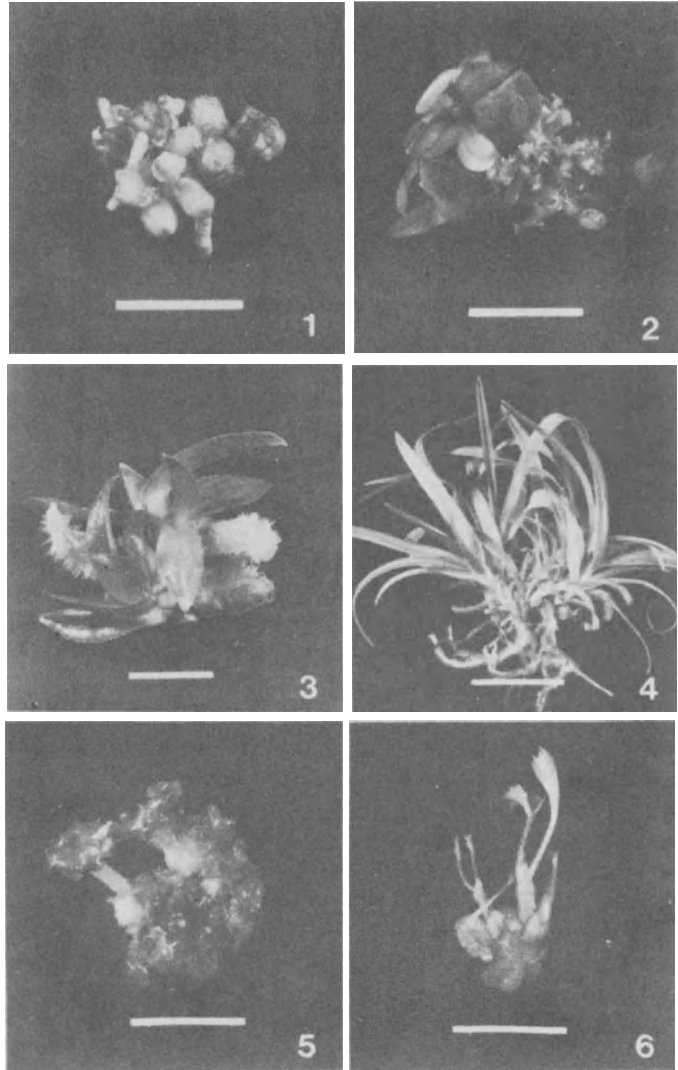
6.1.8 Orchids

Dr. RAO has written about most orchids (Chap. I.3 of this Vol.). Although the commercial production experience of Twyford Laboratories has been with *Cymbidium*, *Cattleya*, *Miltonia*, *Odontoglossum*, Vandaceous types and *Phalaenopsis*, and the various intergeneric crosses of each group, this article restricts its comments to the genera *Phalaenopsis* and *Paphiopedilum*. Both genera are important as cut flowers and pot plants, although the economic value of *Paphiopedilum* is many times greater than that of *Phalaenopsis* which has at present relatively little standing in Europe. It is generally believed that both genera are difficult to propagate. The *Paphiopedilum* has great value both as a cut flower and a pot plant, but there tends to be substantial variation and significant waste in seed propagation. Careful clonal selection and propagation would eliminate such

waste, with an increase in overall efficiency and quality for the cut flower and pot plant producer. Similar arguments are true for *Phalaenopsis*, which is a monopodial and, therefore, unlike the sympodial orchids, has few suitable buds for culture and examination, without causing irreparable damage. The peduncle has 3 or 4 buds covered by scale leaves, each capable of giving rise to a flower spike, or a shoot if removed from the plant as a stem cutting and grown in vitro. Anatomical examination demonstrates the presence of a vegetative and floral meristem in these buds. The careful dissection of the bud and culture can very rapidly result in a small plant and modification of these conditions in several small shoots (Fig. 1–6). Further study of this plant demonstrates several phenomena found by workers in plants of other genera, namely the production of plantlets from the leaf lamina. Unlike many other commercial growers, no problem has been experienced during the studies of this genera in producing the embryonic protocorm structure (Figs. 1–6) and the utilisation of these for propagation purposes in media, similar to those used for *Cymbidium* (MARSTON and VORAURAI, 1967), although proliferation of protocorms on solid media was better than in liquid. In all cases the progeny so produced have flowered with the characteristics of the donor plants, generally within 16 months from removal of in vitro culture. In practice, plants substantially larger than those normally produced by aseptic seed culture, can be produced in any quantity by the tissue-culture technique.

Many workers have tried to multiply the genus *Paphiopedilum* using various parts of the plant, including leaf tips, apical shoot tips, root tips and flower buds. The normal result of such attempts is death of the explant for one of three reasons: (1) the inadequacy of the media, (2) swamping with bacteria or fungi, a result of inadequate surface sterilization, (3) the carry-over of systemic organisms contained within the explant or contaminating the explant from the viscous exudate from cut leaf bases and stem tissue during dissection. Many years of experimentation by several workers in the author's laboratory, have resulted in some significant advances. First, sterilization techniques have been developed which generally reduce losses due to contamination. Normal surface sterilization with hypochlorite solution is followed by a subsequent surface sterilization of the well-exposed explant (usually an apical tip) prior to excision, using 70% alcohol, and 1% mercuric chloride, followed by copious washing with sterile water. This technique reduces death by bacterial and fungal colonies to practically nil, although some tissue is killed if the mercuric chloride is left too long or inadequately leached. The duration of the mercuric chloride treatment must be found by experimentation as it must be related to the extent and nature of the contaminating organisms and resistance of the tissue, but periods of less than 60 sec have been successful with seedlings of several crosses.

During media trials for *Paphiopedilum*, work on a closely related genus, *Phragmipedium*, was initiated and a system for the routine in vitro propagation established (STOKES *et al.*, 1975). The application of this technique to *Paphiopedilum* is successful in that a definite, although compared to *Cymbidium*, slow propagation of selected clones can be achieved. It has, however, proved impossible to cause explants to produce distinct protocorms and until this is achieved the multiplication rate will be slow. As with all orchids, there is a great danger of increasing the distribution of virus via tissue culture of these genera. To prevent



Figs. 1–6. Various plantlets produced via tissue culture on modified MURASHIGE and SKOOG (1962) media. Fig. 1. Protocorms of *Phalaenopsis* Bridesmaid Purity on modified MURASHIGE and SKOOG media plus 5 mg/l BAP. Fig. 2. Plantlets of *Phalaenopsis* Bridesmaid Purity on modified MURASHIGE and SKOOG media, no growth substances. Fig. 3. *Paphiopedilum* proliferations on modified MURASHIGE and SKOOG media +0.5 mg/l BAP +1.0 mg/l NAA. Fig. 4. Plantlets *Phragmipedium sedenii* on modified MURASHIGE and SKOOG media with 0.05 mg/l zeatin. Fig. 5. Shoot formation in *Begonia* (tuberous hybrid) media modified MURASHIGE and SKOOG +1 mg/l BAP. Fig. 6. Shoot formation in *Pelargonium hortorum* from callus on modified MURASHIGE and SKOOG +0.5 mg/l BAP +0.1 mg/l NAA +1.0 mg/l gibberellic acid. Scale line = 1 cm

irreparable damage to the orchid industry it is imperative that only disease-free plants are produced and distributed—the basic aim of MOREL'S (1960) original work. The dangers with *Phalaenopsis* and *Paphiopedilum* are as great as those with *Cattleya*, *Cymbidium* and *Vanda*, and, therefore, only plants which carry suitable test certificates should be purchased by growers.

6.1.9 Araceae

Anthurium andreanum produces a long-lasting cut flower which is popular in the European and American markets. The area under cultivation throughout the world has increased remarkably during the past five years. Some statistics are presented in Table 4. At present all plants are produced from seed. Berries take 6-7 months to ripen after fertilization and must be sown immediately. The juvenile phase of the plant may last up to three years.

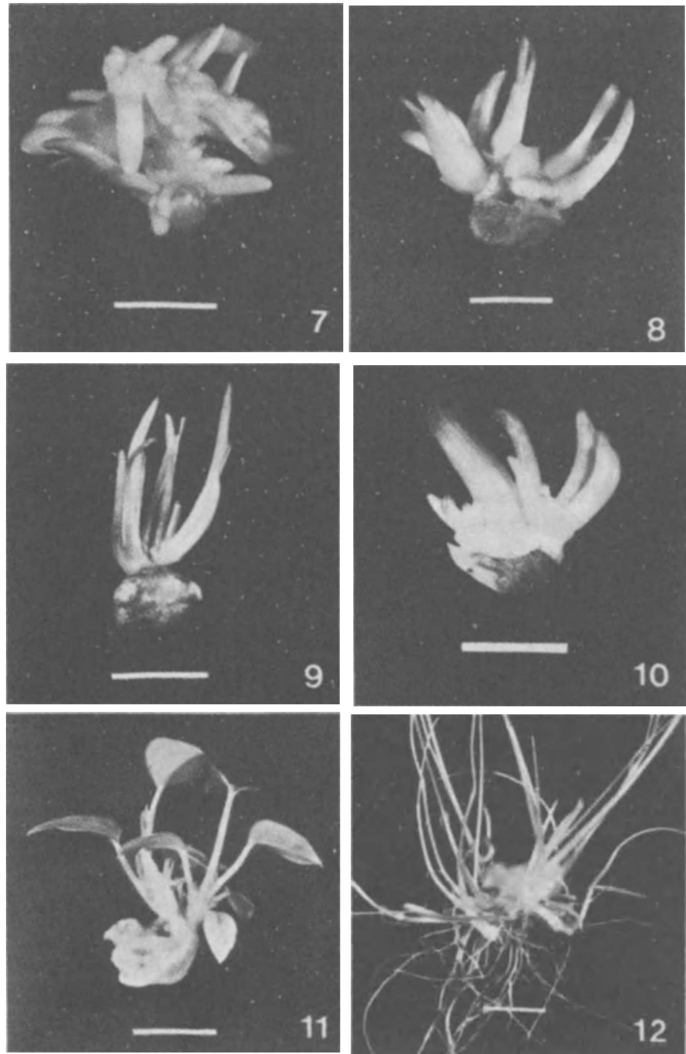
The subsequent flower crop exhibits great variability in flower size, quality, colour and yield, as expected from an outbreeding plant. Attempts to find a satisfactory method for vegetative propagation have failed. PIERIK *et al.* (1974) have produced plants from callus derived from embryos, and young soft tissue of a small number of adult plants. Callus growth was achieved on a modified MURASHIGE and SKOOG media (1962) in the presence of 1 – 5 mg/l-PBA [(6-benzylamino)-9-(7-tetrahydropyranyl) 9 H-purine.] and in the case of adult tissue with 1 mg/l 2,4-D.

Experiments were performed in the dark at 25° C. Shoots could be produced in the dark and at an enhanced rate in the light.

A more generally applicable method of propagation was discovered in 1974 (unpublished results) during research in the author's laboratory on a range of genotypes of every age. Only three adult genotypes, representing a low percentage, failed to grow on modified MURASHIGE and SKOOG (1962) media with no auxin at 25° C in a 16-h photoperiod. The system has been developed for commercial production work but explants must be restricted to meristems not exceeding 0.75 × 1.0 mm in size. Although initial growth rates are very slow, no contamination by systemic organisms has been experienced. The commercial production of selected clones of *A. andreanum* was started at Twyford Laboratories in November 1974, and subject to final trials, plants should be available at general grower level in 1977 with first commercial flowering probably in 1979. This time delay from start to first income is experienced with most plants to which tissue culture is applied but there is no way to shorten the period to obtain cash returns on the significant investment made. The application is economically important because it will enable the specialist breeder to make available the many beautiful plants and exciting novelties hitherto seen by relatively few. The technique has been tested and proved satisfactory on genotypes of *A. scherzerianum* and production was started in July 1975.

6.1.10 Other Monocotyledons

Many workers have now achieved in vitro propagation of a range of genera in several families of the monocotyledons (Figs. 7–12). HUSSEY (John Innes Research Institute, Norwich) has demonstrated (see HUSSEY and WYVILL, 1972, 1973; HUS-



Figs. 7–12. Shoot formation in various monocotyledons produced on modified MURASHIGE and SKOOG (1962) media. Fig. 7. Shoot formation from basal plate tissue of *Narcissus*. Fig. 8. Shoot formation from basal plate tissue of *Nerine bowdenii*. Fig. 9. Shoot formation from bulb scale of *Lilium*, sp. Fig. 10. Shoot formation from bulb scale of *Hyacinthus*, sp. Fig. 11. Shoot formation from callus of *Anthurium andreanum*. Fig. 12. Shoot formation from callus of *Gladiolus*

SEY, 1974) in a comparative study that some 12 hybrids belonging to Iridaceae, Liliaceae and Amaryllidaceae can be propagated in vitro. Plantlets could be induced directly from stem tissue without intervening callus tissue in 9 species. In the case of *Gladiolus*, *Hyacinthus*, *Spiraxis*, low concentrations of auxin were required to stimulate plantlet formation on stem tissue, but no plantlets could be induced on the stem tissue of *Freesia*, *Narcissus* or *Tulipa*. Plantlets could be

induced on pieces of bulb or corm tissue from all species tested but such material proved difficult to free from bacterial and fungal contaminants.

Callus could be obtained from all species except *Tulipa* and *Hippeastrum;* in *Narcissus* it could only be formed from ovary tissue. Plantlets could be formed from the callus at zero or very low auxin concentrations and could be stopped as the concentration of auxin was increased. This occurred in all species tested, except *Gladiolus*, *Spiraxis* and *Schizostylis*. HUSSEY concluded that there was no simple relation between the natural rate of vegetative increase and that obtained in vitro. Once plantlets had been produced, they could be further multiplied by splitting the basal meristem. The divided plants grew normally on subculture and could be split repeatedly at intervals of 6–10 weeks.

ZIV *et al.* (1970) have produced callus and plantlets directly from 3–4 mm sections of inflorescence stem explants of *Gladiolus* in the presence of high levels of NAA. By further culturing the developing bud, clumps of plantlets with cormlets were produced giving a very substantial rate of multiplication. GINZBURG and ZIV (1973) demonstrated that kinetin stimulates both shoot and cormel production, while SIMONSEN and HILDEBRANDT (1971) regenerated virus-symptomless plants from cormel stem tips. Similar procedures have been employed at Twyford Laboratories, together with cormlet explants, and although a scheme for satisfactory plantlet production can be developed, no method for exclusive cormlet initiation has been found, thus any production procedure results in cormlets of variable sizes. It is now feasible to produce foundation stocks of virus-indexed *Gladiolus* cormlets, on a commercial scale.

BRANTS (1968) has produced a medium which enables 80% of meristems from *Freesia* to grow and has demonstrated that 75% of plants produced, react negatively in serological tests for *Freesia* mosaic virus and *Phaseolus* Virus 2. The in vitro propagation of *Freesia* presents no problems, it being possible to bring about an adequate rate from almost any part of the plant including the corm and flower stem, or by slight modification of the work described by BRANTS (1968). The patent applications for a method of propagation of *Freesia* based on the work of the John Innes Research Institute, is effectively the same as that described by ZIV *et al.* (1970) for *Gladiolus*, but is only one of a number of methods (BAJAJ and PIERIK, 1974; HOLDGATE *et al.*, 1975).

Again cormlet production on the regenerated plants occurs readily in vitro. The cormlets can be harvested, stored and subsequently incorporated into a commercial flower-producing regime. Growing trials have so far demonstrated the production of slightly earlier uniform high-quality flowers.

The work on *Gladiolus*, resembles in many respects the earlier work published by SHERIDAN (1968) on Lily. SHERIDAN (1968, 1974) had demonstrated that *Lilium longiflorum* plants from tissue culture flower true to type. In general, chromosome stability was maintained, although one tetraploid plant and one trisomic callus was found. It is now well known that *Lily* can be produced in vitro via callus or from bulb scales, both flowering true to type. By making a number of modifications, it has been possible to fit *Lily* into a routine production schedule at Twyford Laboratories. Plants can be muliplied rapidly and induced to produce small bulbs which can be stored or planted directly.

Work during the past few years on various plants which have bulbs or corms as storage organs, demonstrates very similar results in their in vitro behaviour. It is clear that regeneration can be effected via callus initiated from a range of tissue of the parent plant or by direct adventitious bud development from flower stem, leaf base or storage organ. Plants which start to develop in vitro can be removed, divided and subcultured with further proliferation. In most cases, plantlets and small storage organs can be formed and although development of the latter can be controlled (as in *Hyacinthus* and *Lily*) the production may be irregular (*Gladiolus* and *Freesia*). The rates of growth and efficiency of plantlet production tend to vary with the conditions being employed which are apparently not individually critical, although following general principles in cytokinin/auxin ratios. The suggestion by PIERIK and coworkers (PIERIK and WOETS, 1971; PIERIK and RUIBING, 1973) that 7 factors (i.e. polarity, origin, length, mineral nutrients, temperature, light and IAA) are effective in bulblet regeneration and growth in *Hyacinthus*, could well partly apply to most other genera. However, the arrangement, combination and variation of these specific conditions, appears limitless. The essential for the tissue-culture laboratory is to find a combination of conditions which will provide an economic rate of propagation without causing variation in plant form. It is unfortunate that many of the important monocotyledons, including *Narcissus*, *Nerine*, and *Gladiolus*, take several years to flower and, therefore, experiments relating to a range of in vitro cultural conditions and the eventual effect, if any, on plant growth and flower quality, are time-consuming and invariably expensive and difficult for the tissue-culture laboratory to perform. The trials performed in conjunction with growers have resulted in the uniform flowering of *Freesia*, *Gladiolus* and *Lily* and many orchids are sufficiently encouraging to anticipate that in other genera, no variation will be experienced if mild auxin and cytokinin treatments are given at the in vitro stage.

Despite the fact that the initiation of callus cultures of monocotyledons was not achieved until substantially after that for dicotyledonous plants, the commercial importance of the former have resulted in a wider application and capability for their commercial propagation in vitro.

6.1.11 Dicotyledons

Work on dicotyledons has not been ignored, and successful applications have been made with *Begonia*, *Pelargonium*, *Chrysanthemum* and *Saintpaulia*. *Pelargonium* can be propagated via calli (PILLAI and HILDEBRANDT, 1968, 1969). Shoot formation from callus occurs on media such as MURASHIGE and SKOOG'S (1962) with the addition of high kinetin (10 mg/l) and low auxin (0.03 mg/l) (PILLAI and HILDEBRANDT, 1969). A similar medium used by Twyford Laboratories enables multiple production of buds although a lower concentration of cytokinin (0.5 mg/l) has been found advantageous. A major problem with *Pelargonium* is that hormonal requirements vary from variety to variety (PILLAI and HILDEBRANDT, 1969) and even using apparent optimal hormone concentrations, variations in multiplication rates still exist; furthermore, callus progressively loses its regenerative capacity. This observation has been confirmed by HOLDGATE *et al.* (1975) but if regenerating callus is continuously removed from the rest of the callus-containing

regenerating tissue, then organogenesis can be maintained over long periods, although the rates are slow and expensive. It has also been found that medium containing low concentrations of auxin (e.g. 0.1 mg/l) are beneficial because under these conditions, nonregenerating callus grows slowly. No genetical variation has yet been observed at the Twyford Laboratories in plants produced in culture and PILLAI and HILDEBRANDT (1968, 1969) make no mention of any variation.

The recent work by FONNESBECH (1974 a) on *Begonia* × *cheimantha* and at Twyford Laboratories on the tuberous *Begonia* and Begonia Rieger Elatior have demonstrated that the in vitro propagation and removal of the bacteria which cause problems in conventional methods of propagation, can be achieved. The important steps in the production and maintenance of disease-free stock plants of *Begonia* has been made. FONNESBECH (1974 a) has been able to correlate the in vitro conditions with those required during the traditional methods of propagation. The system is markedly affected by temperature, which is optimal for shoot formation at 18–21° C and inhibiting above 24° C. The work of FONNESBECH (1974 b) and at Twyford Laboratories, demonstrates that shoot formation is strongly dependent on the presence of cytokinin, while auxins have only a modifying effect on the kinin influence and auxin is frequently not required at all. These results do not appear to agree with those of RINGE and NITSCH (1968) who, to obtain shoots in *Begonia*, required the presence of auxin. In Fonnesbech's work, optimal concentrations of BA and NAA were 0.5 to 1.0 mg/l and 0.01 mg/l respectively, but Fonnesbech points out that temperature has an overriding effect. Increases in the concentration of cytokinin treatment prompts further shoot formation, but the leaves were frequently malformed. Lower concentrations of cytokinin failed to stimulate significant shoot formation.

Flowering trials with dicotyledons produced in vitro have shown great uniformity and trueness to type, although in the case of Pelargoniums there is a tendency for virus-free plants to produce very large leaves, which can be controlled by modifying the conditions of growth.

7. Conclusions

Very careful background research work is required before any tissue culture laboratory can start to propagate a selected plant, perhaps the only one of its kind, by in vitro techniques. Slight variations in conditions for sterilization and in culture media can affect results both qualitatively and quantitatively. Similarly the environmental conditions employed should be carefully controlled and particular attention given to temperatures and light sources.

The future application of tissue culture in horticulture is very much dependent on recognising capabilities and communication between growers and those concerned with the application of this technology. Growers and breeders may be aware of the more dramatic claims involving the production of new types of plants via genetic engineering but few are aware of the significant advances which have been made in the potential of tissue culture as a tool for improving the performance of existing crops.

Communication must not be such as to stimulate the wide proliferation of small, isolated or commercially nonviable tissue culture laboratories operated by unqualified staff. It would be unwise for others to follow the example of some orchid growers in creating their own backroom laboratories without the backup research and testing facilities essential to overcome major production problems and to provide disease-free or indexed stock. The tissue culture laboratory requires a substantial capital investment for providing a complete and adequate service to each individual customer. Although there have been enormous advances in capability during the past five years, significant areas of research, pertinent to plant production, have been ignored. These omissions may be caused by lack of finance, or by being outside the interests of many scientists, or by failure of communication and mutual understanding between workers. It is important to identify where advances are required and to develop appropriate techniques.

It would appear that the desirable advances can only be achieved by active cooperation at three levels involving Government-financed research institutes and university researchers, commercial laboratories and the breeder/growers. Government-financed and university laboratories generally have the facilities to perform the essential fundamental research. It is unlikely that the commercial laboratory will be able to devote expenditure on all essential fundamental problems, as its work must be very much one of compromise, balancing the essential basic development and production research and facilities to ensure the creation of uniform plants in the quantity and at the time required by the grower. In most cases the potential return which tissue culture can achieve for the grower/breeder appears to be sufficient to encourage an investment in the form of a cooperative approach. Unless there is substantial grower involvement, at least to a level which will enable a logical commercial assessment to be made on the true performance of individual plants selected by the breeder, progress during the next few years will be slow. Some cooperative relationships already exist between Twyford Laboratories and a few leading breeders with whom essential trials are being performed on *Anthurium*, *Begonia*, *Freesia*, *Gladiolus*, *Lily*, *Nerine*, *Pelargonium* and *Saintpaulia*, besides many orchids, but obviously more cooperative work is required, particularly in evaluating the mass propagation and growing of the disease-free cultivars which exist. What can be achieved at the research bench is of no practical or financial benefit to the industry until it is employed on a day-to-day basis to help improve the efficiency and productivity at the grower level.

References see page 207.

3. Tissue Culture in the Orchid Industry

A. N. Rao

1. Introduction

Orchids are distinctive plants. Taxonomically, they represent the most highly evolved family among monocotyledons, with approximately 450 genera and 15000–20000 species; their varieties are described by Holttum (1953) and Purseglove (1972). While orchids are perennial herbs, some are epiphytes or saprophytes (Summerhays, 1951; Holttum, 1953; Campbell, 1964) and although the specialized flower structure conforms to a particular plan, the vegetative parts show great variation. Pollination is a specialized process (Pijl and Dodson, 1966) with pollen organized into massive or sticky type of pollinium. In their natural condition the majority of flowers are not pollinated, their ovules are not fertilized and capsules are rarely formed. Details of orchid embryology and cytology have been compiled and reviewed by Swamy (1949a, b), Maheshwari (1950), and Withner (1959). Of the twenty genera examined so far, the number of seeds produced per capsule is high ranging from 5000–6000 in such genera as *Cephalanthera*, to 2–3 millions in *Cynoches* and *Cattleya* (Salisbury, 1942; Shushan, 1959; Withner, 1959; Jeyanayaghy and Rao, 1966; Arditti, 1967a). Because of their particular fungal requirement, less than 5% of seeds germinate in their natural environment.

Orchids have been well known to horticulturists and herbalists for the past 400 years (Arditti, 1966a; Coats, 1969; Garay, 1974). In the past, explorers employed by royalty and wealthy Europeans, were sent to far-off places to gather orchids. After growing and acclimatizing orchids under local conditions, the plants were sold by auction (Withner, 1959; Burkill, 1964). Epiphytic orchids were the more commonly grown—their natural potential for vegetative propagation giving strong impetus for their cultivation. Most of the cultivated orchids, however, were found to be self-sterile. The seeds sown in nursery beds required long periods of germination and any disturbance to the soil or physical environment destroyed the whole population (Northen, 1962). Nevertheless, interest in the development of new plants of greater horticultural value grew. John Daminy produced the first *Calanthe* hybrid that flowered in 1856 at Veitch Nursery, Exeter, England (Withner, 1959). Following him, many others successfully obtained interspecific and intergeneric hybrids. The Royal Horticultural Society, U.K. is the international official body for registering orchid hybrids.

Plant tissue culture has opened a number of possibilities and the literature has accumulated rapidly over the last 30 years. The application of tissue culture techniques to orchids and the benefits derived, such as production of quality plants in large quantities by clonal multiplication, establishment of hybrid plants,

improvement of orchid trade and industry, are discussed in this article. The reader is also referred to earlier reviews by WITHNER (1959), MARSTON (1966), ARDITTI (1967a) and MURASHIGE (1974).

2. Tissue Culture Techniques and Media

The pioneering work of BERNARD (1909) is important in the development of in vitro culture techniques of orchids. He successfully isolated the root-infecting fungi helpful in orchid seed germination. Comparative germination studies were made of infected and noninfected seeds. When sterilized seeds were grown in association with the fungus, the percentage of germination improved considerably. The importance of balanced nutrition in maintaining a stable relationship between host and endophyte was explained by HARVAIS and HADLEY (1967).

Subsequently, the work of KNUDSON (1922, 1924, 1925) clarified many important points regarding formation of seedlings and organogenesis. Fungi were chiefly responsible for breaking down starch into simple sugars for the germinating seeds. Osmotic concentration of medium and physico-chemical stimuli were either ineffective or only partially helpful in growth promotion. Thus KNUDSON'S work showed, for the first time, that germination of orchid seeds was possible in vitro without fungal association. Certain plants grown in vitro flowered, though the size of the plants was smaller than those grown in vivo (KNUDSON, 1930). KNUDSON (1951) also suggested a medium that provided balanced organic and inorganic nutrition for the developing seedlings. BURGEFF (1936) obtained considerable data regarding seed germination in about 25 genera, and at least that many species or hybrids. The medium proposed by VACIN and WENT (1949) is widely used for germination of hybrid seeds. In the Singapore Botanic Gardens, where the orchid hybridization program has been actively pursued for the last four decades, only the VACIN and WENT medium (1949) has been used. To date, at least 25 media (including MURASHIGE and SKOOG'S medium, 1962) suitable for the growth of orchid seedlings have been proposed and utilized. For detailed composition of such media see WITHNER, 1959; ARDITTI, 1967a; BUTENKO, 1968.

Occasionally a particular recipe is proposed for a given genus or species, and improvised by the addition of various growth adjuncts to the medium, such as coconut milk, tomato juice, banana extract, different fruit juices, fish emulsion, beef extract, and even beer (ALBERTS, 1953; WITHNER, 1959; ARDITTI, 1967a). In many instances such attempts have no doubt yielded beneficial results but the physiology of nutrition of seedlings is difficult to explain. Subsequent to defining the medium, other conditions were outlined in various works: determining the suitable pH; minimizing the fungal infection; sterilization of the inoculum, whether seeds or tissues. Considerable literature was accumulated and discussed by WITHNER (1959). Some of the papers published after this date, and which are relevant to the present topic, are considered here.

In the history of tissue culture, usage of liquid medium is an important landmark. Most of the tissues or organs were grown on agar medium and, generally, liquid medium was better suited for root cultures (STREET, 1969). By the efforts of subsequent workers, the use of a liquid medium became common for callus or

organ culture, in either static or moving positions. In the latter case, a shaker was used which allowed either lateral or gyratory movements, providing ample aeration for growing tissues (HELLER, 1949, 1953; STEWARD *et al.*, 1958; WHITE, 1963).

In earlier studies solid media were used for embryo culture, differentiation of tissues or organogenesis. Research conducted in the last few years, however, indicates liquid medium as more effective for induction of proliferation, particularly in protocorm or callus. Many workers used both types of media to study callus growth and its organogenesis (Table 1). Apical meristems of *Cymbidium* for example, were excised from young shoots and inoculated on a liquid medium. Cultures were agitated for four weeks during which protocorm-like bodies were formed within 2½ months. Many shoots developed in the two-week rest period, which followed; after transfer to an agar medium, numerous plantlets developed (WIMBER, 1963, 1965). A distinction was made between the starting, standard maintenance, and rooting media based on their suitability. *Cattleya* meristems were used as inoculae and the time sequence involved to raise successful culture was given by LINDEMANN (1967). Other requisites to setting up cultures—surface sterilization of seeds, different materials used as inoculae, pH of medium, glass or

Table 1. Orchids used in tissue culture work, media employed and references

Plant species	Media employed	Reference
Acianthus reniformis	Knudson C (A)[a]	MCINTYRE *et al.* (1974)
Aerides odoratum	Vacin and Went (A)	SAGAWA and VALMAYOR (1966) VALMAYOR and SAGAWA (1967)
Aranda	Vacin and Went (LA)[b]	LOH (1975) LOH *et al.* (1975) TEO and TEO (1974)
Arundina bambusifolia	Vacin and Went Knudson C Raghavan and Torrey (A)	MITRA (1971)
Ascofinetia (cherry blossom)	Vacin and Went (LA)	INTUWONG and SAGAWA (1973)
Brassavola cucullata *B. nodosa*	Vacin and Went (A)	SAGAWA and VALMAYOR (1966) VALMAYOR and SAGAWA (1967)
Brassavola perrinii	Vacin and Went (A)	VALMAYOR and SAGAWA (1967)
Broughtonia sanguinea	Vacin and Went (A)	SAGAWA and VALMAYOR (1966) VALMAYOR and SAGAWA (1967)
Caladenia *Caladenia carnea* *Caladenia latifolia* *Caladenia Menziesii*	Knudson C (A)	MCINTYRE *et al.* (1974)
Calanthe	Knudson C (A)	ITO (1967)
Calanthe	Burgeff N_3f (A) Karasawa (A) Knudson C (A) Liddell (A)	KARASAWA (1966)

[a] Agar medium.
[b] Liquid, Agar media.

Table 1 (continued)

Plant species	Media employed	Reference
Calochilus Robertsonii	Knudson C (A)	McIntyre *et al.* (1974)
Calopogan pulchellus	Burgeff N_3f (A) Knudson C (A)	Stoutamire (1964)
Cattleya	Heller (A)	Champagnat *et al.* (1970)
Cattleya	Knudson C (A)	Alberts (1953) Arditti (1966b, c, d) Champagnat and Morel (1969) Farrar (1963) Hirsh (1959) Ito (1955, 1965) Knudson (1951) Lawrence and Arditti (1964) Morel (1965)
Cattleya	Knudson III (A)	Morel (1964)
Cattleya	Burgeff N_3f (A) Karasawa (A) Knudson C (A) Liddell (A)	Karasawa (1966)
Cattleya	Lindemann, Gunckel and Davidson (A)	Lindemann *et al.* (1970)
Cattleya	Livingston (A)	Noggle and Wynd (1943)
Cattleya	Princeton Gel (A)	Rossiter (1960)
Cattleya	Vacin and Went (LA) Morel (LA)	Scully (1967)
Cattleya	Kyoto solution (A)	Tsukamoto *et al.* (1963)
Cattleya	Knudson C (A) Vacin and Went (A)	Valmayor (1974)
Cattleya	Knudson C (A) Murashige and Skoog (A) Vacin and Went (A)	Ziegler *et al.* (1967)
Cattleya aurantiaca	Knudson C (A)	Arditti (1968)
Cattleya aurantiaca	Knudson C (A) Heller (L)	Pierik and Steegmans (1972)
Cattleya elongata	Vacin and Went (A)	Sagawa and Valmayor (1966) Valmayor and Sagawa (1967)
Cattleya labiata	Spoerl (A)	Raghavan (1964) Raghavan and Torrey (1964)
Cattleya violacea	Vacin and Went (A)	Sagawa and Valmayor (1966) Valmayor and Sagawa (1967)
Cattleya bowringiana	Vacin and Went (LA) Morel (LA)	Scully (1967)
	Vacin and Went (A)	Sagawa and Valmayor (1966) Valmayor and Sagawa (1967)
Cattleya loddigesii	Vacin and Went (A)	Sagawa and Valmayor (1966) Valmayor and Sagawa (1967)

Table 1 (continued)

Plant species	Media employed	Reference
Cattleya portia	Knudson B (A)	Knudson (1925)
	Vacin and Went (A)	Valmayor and Sagawa (1967)
Cattleya skinnerii	Knudson C (A)	Knudson (1951)
	Vacin and Went (LA) Morel (LA)	Scully (1967)
Cymbidium	Knudson C (A)	Champagnat *et al.* (1966)
	Knudson III	Morel (1964)
	White (LA)	Steward and Mapes (1971)
	Knudson C (LA) Vacin and Went (LA)	Thompson (1971)
	Kyoto solution (A)	Tsukamoto *et al.* (1963)
	Knudson (LA)	Morel (1965) Wilfret (1966)
	Tsuchiya (L)	Wimber (1963, 1965) Wimber and Van Colt (1966)
Cymbidium ensifolium *Cymbidium goeringii*	Knudson C (A)	Ueda and Torikata (1968, 1969a, b, 1970a, b, c, 1972)
Cymbidium gyokuchim	Hyponex (A)	Kano (1968)
Cymbidium insigne *C. kauran, C. pumilum* *C. tracyanum*	Knudson C (A)	Ueda and Torikata (1968, 1969a, b, 1970a, b, c, 1972)
Cymbidium virescens	Knudson (A)	Champagnat *et al.* (1968)
C. Cygnus "Inghans"	Knudson C (A)	Fonnesbech (1972)
Cypripedium	Knudson (LA)	Hegarty (1955)
Cypripedium acaule	Hyponex (A)	Kano (1968)
Cypripedium acaule	Knudson C (A) Burgeff N_3f (A)	Stoutamire (1964)
Cypripedium reginae	Knudson C (A) Burgeff N_3f (A)	Stoutamire (1964)
Cypripedium Christopher *Cyp.* Manda, *Cyp.* Van Dyck	Liddell (A)	Liddell (1953)
Dendrobium	Knudson C (A)	Valmayor (1974)
Dendrobium	Vacin and Went (A)	Gilliland (1958) Nimoto and Sagawa (1961) Valmayor and Sagawa (1967) Valmayor (1974)
Dendrobium	Marsten (A)	Marston (1966)
Dendrobium nobile	Vacin and Went (A)	Sagawa and Valmayor (1966) Valmayor and Sagawa (1967)
	Knudson C (LA)	Ito (1955) Sagawa and Shoji (1967)
	Burgeff N_3f (A) Karasawa (A) Knudson C (A) Liddell (A)	Karasawa (1966)
Dendrobium pulcherrima	Vacin and Went (A)	Sagawa and Valmayor (1966) Valmayor and Sagawa (1967)

Table 1 (continued)

Plant species	Media employed	Reference
Dendrobium Bali	Vacin and Went (A)	SAGAWA and VALMAYOR (1966) VALMAYOR and SAGAWA (1967)
Dendrobium Clara Cooper	Vacin and Went (A)	SAGAWA and VALMAYOR (1966) VALMAYOR and SAGAWA (1967)
Dendrobium Jaquelyn Thomas	Vacin and Went (A)	ISRAEL (1963) SAGAWA and SHOJI (1967) SINGH and SAGAWA (1972) VALMAYOR and SAGAWA (1967)
Dendrobium May Neal *Dendrobium* pampador	Knudson B (A)	VAJRABHAYA and VAJRABHAYA (1974)
Dendrobium Phalaenopsis	Vacin and Went (LA)	KIM *et al.* (1970)
Dendrobium spalding *Dendrobium* Thomas	Vacin and Went (A)	SAGAWA and VALMAYOR (1966)
Dendrobium uniwai crystal	Vacin and Went (A)	SANGUTHAI *et al.* (1973)
Dipodium	Vacin and Went (A)	McINTYRE *et al.* (1974)
Diuria *Diuria punctata*	Vacin and Went (A)	McINTYRE *et al.* (1974)
Doritis pulcherrima	Vacin and Went (A)	SAGAWA and VALMAYOR (1966) VALMAYOR and SAGAWA (1967)
Encyclia tampensis	Knudson B	FREI (1973) FREI *et al.* (1975)
Epidendrum	Murashige and Skoog (A) Knudson C (A)	CHURCHILL *et al.* (1970)
Epidendrum atropurpureum	Vacin and Went (A)	SAGAWA and VALMAYOR (1966) VALMAYOR and SAGAWA (1967)
Epidendrum fragrans *E. tampense*	Vacin and Went (A)	VALMAYOR and SAGAWA (1967)
Epidendrum Bumble Bee	Vacin and Went (A)	VALMAYOR and SAGAWA (1967)
Epidendrum cv. O'Brienianum	Heller (LA) Knudson (LA) 'M' medium (LA) Murashige and Skoog (LA)	CHURCHILL *et al.* (1970, 1973) CHURCHILL *et al.* (1971) RUDOLPH *et al.* (1972)
	Ojima and Fujiwara (A)	CHURCHILL *et al.* (1972)
Eriochilus cucullatus	Knudson C (A)	McINTYRE *et al.* (1974)
Glossodia major	Knudson C (A)	McINTYRE *et al.* (1974)
Goodyera pubescens *G. oblongifolia*	Knudson C (A) Burgeff N_3f (A)	STOUTAMIRE (1964)
Habenaria ciliaris *H. blephariglottis*	Knudson C (A) Burgeff N_3f (A)	STOUTAMIRE (1964)
Laeliocattleya	Pfeffer (A)	KNUDSON (1930)
Laeliocattleya 'Ibbie'	Knudson C (A)	ARDITTI (1967b)
Laeliocattleya Portia Mayflower	Heller (LA) Knudson 'M' medium Murashige and Skoog	CHURCHILL *et al.* (1971) CHURCHILL *et al.* (1970, 1973)
Liparis loeselii	Burgeff N_3f (A) Knudson C (A)	STOUTAMIRE (1964)

Table 1 (continued)

Plant species	Media employed	Reference
Listera cordata	Burgeff N_3f (A) Knudson C (A)	STOUTAMIRE (1964)
Lycaste	Knudson C (LA)	MOREL (1965)
Microtis sp.	Knudson C (A)	MCINTYRE *et al.* (1974)
Microtis bipulvinaris	Knudson C (A)	MCINTYRE (1974)
Microtis oblonga	Knudson C (A)	MCINTYRE (1974)
Microtis unifolia	Knudson C (A)	MCINTYRE (1974)
Miltonia	Burgeff N_3f (A) Karasawa (A) Knudson C (A) Liddell (A)	KARASAWA (1966)
	Knudson III (A)	MOREL (1964)
	Knudson C (LA)	MOREL (1965)
Neostylis Lau Sneary	Vacin and Went (LA)	INTUWONG and SAGAWA (1973)
Odontoglossum	Knudson C (LA)	MOREL (1965)
Oncidium ampliatum *O. haematochilum* *O. luridum* *O. stipitatum*	Vacin and Went (A)	SAGAWA and VALMAYOR (1966) VALMAYOR and SAGAWA (1967)
Oncidium Mem. Pepita de Restrepo.	Vacin and Went (A)	VALMAYOR and SAGAWA (1967)
Paphiopedilum	Knudson C (A)	ERNST *et al.* (1970)
	Burgeff N_3f (A) Karasawa (A) Knudson C (A) Liddell (A)	KARASAWA (1966)
Paphiopedilum cv. Winston Churchill	Thomale GD (A)	ERNST (1974)
Phalaenopsis	Knudson C (A)	ERNST (1967a, b, 1975) ERNST *et al.* (1970) SAGAWA (1961)
	Vacin and Went (A)	INTUWONG *et al.* (1972) KOTOMORI and MURASHIGE (1965) SCULLY (1965, 1966)
	Burgeff N_3f (A) Karasawa (A) Knudson C (A) Lidell (A)	KARASAWA (1966)
	Difco orchid agar (A)	NORTHEN (1953)
	Knudson C (A) Murashige and Skoog (A)	TSE *et al.* (1971)
Phalaenopsis amabilis	Knudson C (A)	ROTOR (1949) VALMAYOR (1974)
	Vacin and Went (A)	VALMAYOR (1974)
Phalaenopsis pulcherrima	Vacin and Went (A)	SAGAWA and VALMAYOR (1966)
Phalaenopsis Chieftani *Phalaenopsis* Elizabethae	Vacin and Went (A)	SAGAWA and VALMAYOR (1966)

Table 1 (continued)

Plant species	Media employed	Reference
Phalaenopsis Doris	Knudson C (LA)	ARDITTI *et al.* (1972)
	Vacin and Went (A)	SAGAWA and VALMAYOR (1966)
Phalaenopsis sunfidor	Vacin and Went (LA)	INTUWONG and SAGAWA (1974)
Pogonia ophioglossoides	Knudson C (A) Burgeff N_3f (A)	STOUTAMIRE (1964)
Pterostylis acuminata *P. concinna*, *P. curta*, *P. nutans*, *P. obtusa*, *P. parviflora*, *P. pedunculata*	Knudson C (A)	MCINTYRE *et al.* (1974)
Renanthera chandler	Vacin and Went (A)	SAGAWA and VALMAYOR (1966)
Rhynchostylis gigantea	Vajrabhaya and Vajrarabhaya (A)	VAJRABHAYA and VAJRABHAYA (1970)
Schomburgkia superbiens	Morel (LA) Vacin and Went (LA)	SCULLY (1967)
Sophrolaeliocattleya	Knudson C (A)	ERNST *et al.* (1970)
Spathoglottis plicata	Vacin and Went (A)	BEECHEY (1970a, b)
Spiranthes cernua	Burgeff N_3f (A) Knudson C (A)	STOUTAMIRE (1964)
Thelymitra	Knudson C (A)	MCINTYRE *et al.* (1974)
Thelymitra aristata *T. carnea*, *T. chasmogama*, *T. ixioides*, *T. nuda*, *T. pauciflora*	Knudson C (A)	MCINTYRE *et al.* (1974)
Vanda	Vacin and Went (A)	SAGAWA and VALMAYOR (1966)
	Vacin and Went (LA)	TEO *et al.* (1973)
Vanda Joaquim	Vacin and Went (A)	GOH (1970a, 1971) ONG (1969) RAO (1963, 1964) RAO and AVADHANI (1963, 1964) SAGAWA and SEHGAL (1967) SINHA (1973)
	White (A)	GOH (1970b)
	Vacin and Went (LA)	KUNISAKI *et al.* (1972)
Vanda Kuniko Sugihara	Vacin and Went (LA)	SANGUTHAI and SAGAWA (1973a)
Vanda Patricia Low	Vacin and Went (A)	SANGUTHAI and SAGAWA (1973a)
Vanda Sanderiana	Knudson C (A) Vacin and Went (A)	VALMAYOR (1974)
Vanilla fragrans *V. phaeantha*	Burgeff N_3f (A)	WITHNER (1955)
V. planifolia	Knudson (LA)	HEGARTY (1955)
V. pompona	Burgeff N_3f (A)	WITHNER (1955)

plasticware used have been well discussed and need not be covered again (WITHNER, 1959; RAO and AVADHANI, 1964; GOH, 1971; TIEMANN, 1971). The effects of photoperiod, light intensity and/or quality, temperature, pH, auxins, vitamins and growth additives on germinating orchid seeds and seedlings, have also been summarized (ARDITTI, 1967a). Soaking seeds for a prolonged period, for example, gave beneficial results in *Cymbidium* (KANO, 1971).

3. Seed and Embryo Culture

After the successful studies of KNUDSON (1951) on *Cymbidium* were published, many workers followed his techniques and started to grow orchid seedlings under laboratory conditions in flasks and test tubes. These studies were undertaken either to trace the seedling development from undifferentiated embryos, or to obtain seedlings for propagation of precious hybrids which had trade and commercial potentialities. By using this well-established procedure, it was also possible to germinate seeds of intervarietal, interspecific or intergeneric hybrids, which otherwise would not normally germinate. Seedlings thus obtained were transferred to community pots for further cultivation to mature plants and were sold either as whole plants or cut flowers. Publications on orchid embryo and tissue culture prior to 1959 have been included in a scientific survey on orchids (WITHNER, 1959).

3.1 Embryo Culture and Hybrids

The following example illustrates the horticulturists' view of the benefits derived from embryo culture. Orchid embryo culture was initiated at Singapore Botanic Gardens in 1928, and to date 1431 hybrids and 142 selfed-species have been successfully produced; of these, 204 have been registered. In Singapore so far, 1182 hybrids have been registered, out of which 84 are from the Botanical Gardens. Without exception the embryo culture method was used in each of them to obtain plantlets and to grow them till they were ready for transferring to community pots. Of these, 4 hybrids, namely *Aranda* Maggie Oei, *Aranda* Wendy Scott, *Aranthera* James Storie, and *Oncidium goldiana*, form the backbone of the cut flower industry in Singapore, the economic implications of which are discussed in another section of this paper. Tissue culture has been practised since 1949 by a few nurseries and orchid enthusiasts and for the production of hybrids (BURKILL, 1964; ITO, 1967; ALPHONSO, 1975). It is estimated that from 1893 to 1974 approximately 106,410 orchid hybrids, produced in different parts of the world, have been registered, and probably 80–85% were developed by the application of tissue culture techniques to hybrid plants.

Variations seen in the seed germination under in vitro and in vivo conditions were recorded in *Cypripedium acaule* and *C. reginae*. CURTIS (1943) and CLEMENT (1973a, b) stressed poor growth rate and survival of these seedlings under natural

conditions. Ecological and physical factors that would promote or inhibit seed germination or non-viability of seeds in 25 orchid species from the Great Lakes area, USA were discussed by STOUTAMIRE (1964).

3.2 Histogenesis and Organogenesis

Details of seedling development, from the perspective of histogenesis and organogenesis, have been studied in very few orchids, although a considerable number of publications are available describing the exomorphic characters of protocorms and seedlings (see SHUSHAN, 1959; ARDITTI, 1966a). It is well known that orchid embryos are small, undifferentiated, with slight or no variations in cell size and arrangement. The epidermal layer becomes more distinct during germination, the cells at the micropylar and chalazal end of the embryo showing a variation in size, arrangement and contents. An increase in cell numbers and size results in the formation of a protocorm. The organization of promeristem may be in the apical region of the embryo or may develop laterally (RAO, 1964; RAO and AVADHANI, 1964). At the region of promeristem, a small notch appears, the inception of a shoot apex. The usual histogenic layers at a shoot apex become distinct when the next stage of development, i.e. leaf primordia is reached. The root develops endogenously in either the middle or basal portion of the protocorm after the formation of a few leaves. The establishment of polarity, and the development of tissues and organs in the embryo are explained in certain genera (RAO, 1964, 1967; RAO and AVADHANI, 1964; BEECHEY, 1970a, b; MITRA, 1971).

3.3 Germination of Immature Seeds

Various workers have demonstrated that immature seeds can be germinated. Savings in terms of days have been calculated when immature seeds are used; a few examples are given in Table 2.

Other reports are as follows. Different orchids belonging to 11 genera *Aerides*, *Brassavola*, *Broughtonia*, *Cattleya*, *Doritis*, *Epidendrum*, *Oncidium*, *Phalaenopsis*, *Renanthera* and *Vanda*, 19 species and 15 hybrids were self-pollinated to determine whether unripe seeds could be used as inocula to obtain seedlings. Depending on the genus or species studied, the shortest period involved ranged from 40–85 days. As can be expected, abortive ovules showed no growth (SAGAWA and

Table 2. Possibilities of immature seed germination

Genus	Time required for mature seed formation	Time when seeds excised	Reference
Dendrobium	270–360 days	120 days	ITO (1955)
Dendrobium	95 days	69 days	NIMOTO and SAGAWA (1961)
Phalaenopsis	130–150 days	90 days	AYERS (1960)
Vanda	110–120 days	50 days	RAO and AVADHANI (1964), ONG (1969)
Vanilla	150–180 days	60 days	WITHNER (1955)

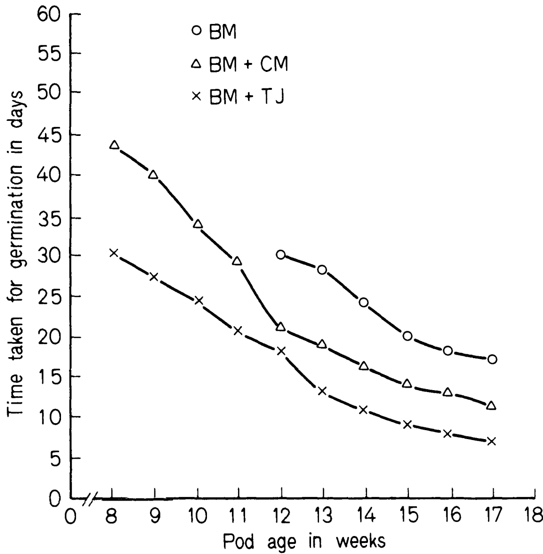
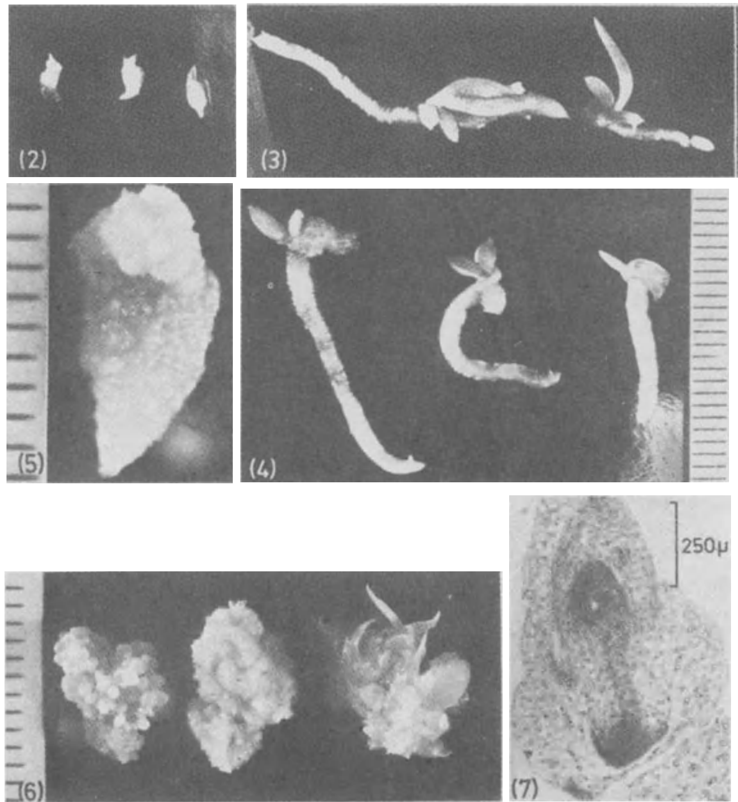


Fig. 1. Germination of immature seeds of *Vanda* Joaquim on different media, BM basal medium, Vacin and Went, CM coconut milk, TJ tomato juice

VALMAYOR, 1966; VALMAYOR and SAGAWA, 1967). Ovaries from pollinated flowers were excised and inoculated on agar medium. After about 11 weeks the ovularies (ovule + placenta) were excised and reinoculated on Vacin and Went's medium supplemented with 1–50 mg/l NAA (optimal concentration 20 mg/l). Seedlings developed from the inoculated tissues. It was claimed that the seedlings developed from placental tissues also, and NAA was essential in the medium. Fertilization occurred 55 days after pollination and possibly the seedlings developed after the completion of embryo development on the agar medium (ISRAEL, 1963). Further detailed studies are required to determine whether placental tissues also undergo embryogenesis. SPOERL (1948) determined the effects of amino acids on the germination of unripe and ripe seeds of *Cattleya*. Onion and yeast extracts promoted immature seed germination in *Dendrobium nobile* (ITO, 1955).

Unripe seeds of *Cattleya aurantiaca* germinated under light conditions and germination was inhibited in darkness. Protocorms were formed in six weeks. When six-week-old cultures were transferred to light from darkness, seed germination progressed showing protocorms. Five-month-old seedlings were transferred into Heller's liquid medium with Na Fe EDTA + sucrose and maintained on a shaker. Cultures were allowed to grow for 10 weeks, and in flasks which contained 10 seedlings, additional protocorms or plantlets were formed; no protocorms were formed in flasks containing only two seedlings. Effects of mass growth or community growth, therefore, were obvious. Different concentration effects of 6-benzylaminopurin were tried on seedlings growing on agar media. Proliferation of tissues or protocorms was obvious at higher concentrations while at lower concentrations seedlings smaller than the control group were formed (PIERIK and STEEGMANS, 1972).

In *Vanda* Joaquim the pod development is completed in 16–17 weeks, producing mature seeds. Seeds excised from 12-week-old pods germinate on basal medium and when the medium is supplemented with tomato juice or coconut milk,



Figs. 2–7. *Vanda* Miss Joaquim. Fig. 2. 22-week-old seedlings on Vacin and Went medium (control). Figs. 3–4. Same on media supplemented with coconut milk and tomato juice respectively. Note the variations in seedling size and root development. Fig. 5. Protocorm showing callus initiation at one end, 8 weeks old. Fig. 6. Different stages of callus development and seedling differentiation in 14-week-old cultures, Control + COM medium. Fig. 7. L. S. seedling showing shoot and root development, 20 weeks old

the immature seeds can be induced to germinate about four to six weeks earlier (Fig. 1). The percentage of germination and seedling size is higher in media with growth adjuncts than in control medium (Figs. 2–4). While 100% seed germination was achieved in 38 days in the control medium, it was much earlier i.e. 14 and 30 days in tomato juice and coconut milk media respectively. Results of such studies are helpful in understanding the germination potential of immature seeds, particularly in those orchids where the pod development takes several months. One need not wait for fruits or seeds to mature, but can obtain quality controlled seedlings in shorter periods of time (RAO and AVADHANI, 1963).

3.4 Callus Development and Abnormal Seedlings

During germination, or under the influence of certain growth regulators, embryos of certain hybrids spontaneously develop into callus masses or abnormal struc-

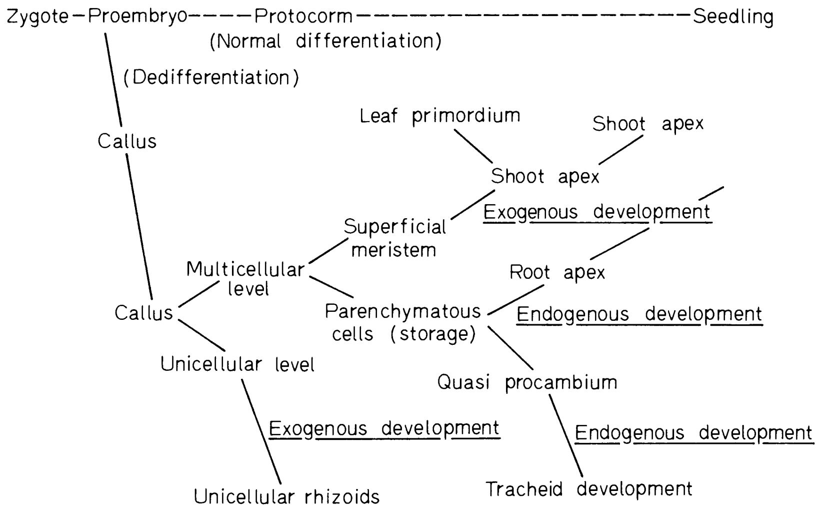


Fig. 8. Schematic representation of organogenesis in orchid callus and seedling formation

tures (CURTIS, 1947; RAO and AVADHANI, 1964; CHAMPAGNAT *et al.*, 1968; GOH, 1970a, b; LOH, 1975). In this laboratory such calli have been noticed in different orchid genera. The callus initiation may be at one end of the protocorm or alternatively the entire protocorm may proliferate into a mass (Figs. 5, 6). On further growth many seedlings are formed from either of the callus masses, and these can be separated and grown as individual seedlings (Figs. 6, 7). Callus induction from embryo can be very useful to obtain an increased number of hybrid plants where the seed production is limited, or the percentage of germination is low (RAO, 1963; RAO and AVADHANI, 1963). Depending upon the medium and growth promoters used, the callus phase can be maintained, or organogenesis can be induced. The differentiation in callus and subsequent developmental changes that lead to plantlet formation are shown in Figs. 8–11 (RAO, 1963).

Proliferation of embryonal tissues and abnormal seedling formation have also been observed. In *Dendrobium* hybrid the basal region of seedlings enlarge into thick thalloid structures. Some of these proliferated dichotomously and each gave rise to a seedling. As many as 20–25 new seedlings developed from an outgrowth of one seedling (GILLILAND, 1958). Abnormal seedlings of *Cattleya* hybrids were formed under the influence of tomato juice. Concentration effects of tomato juice and the presence of sugar were also noted (ARDITTI, 1966c, d).

3.5 Effects of Different Treatments and Growth Adjuncts

Orchid seeds, though small with a thin seed coat, can sometimes withstand prolonged storage at low temperature or treatment with solutions used for surface sterilization (RAO and AVADHANI, 1963; ITO, 1965). Fresh *Phalaenopsis* seeds were soaked in calcium hypochlorite solution and refrigerated for 2–3 days before

sowing. This treatment did not affect percentage germination (NORTHEN, 1953). Different sterilizing solutions alter the rate of seedling development and reflasking of rooted plantlets was difficult. In order to overcome this problem, the relative merits of several sterilizing solutions normally used were tried. A methyl propionic compound (commercial name—Pana drench) solved the problem and uniform *Cattleya* seedlings developed from the surface sterilized seeds (FARRAR, 1963). Protocorms of *Phalaenopsis* and *Epidendrum* were inoculated on Knudson's C agar media supplemented with different surfactants 10–100 mg/l such as Igepon, Neodol, Sulfotex, Turgitol etc. Higher concentrations (1000 mg/l) were inhibitory (ERNST and ARDITTI, 1968).

To assess their growth-promoting properties different fruit extracts were incorporated into the media used in various experiments. Although acetone and ethanol fractions of tomato juice promoted the growth of *Cattleya* seedlings, some seedlings were abnormal. Lower concentrations of tomato juice were recommended for growers (ARDITTI, 1966c, d, 1968). Strained apple juice, along with peptone was found to be effective on seedling development of *Cymbidium* and *Paphiopedilum* (TSUKAMOTO *et al.*, 1963). Sauerkraut juice and vitamin B12 improved the growth of *Cymbidium* seedlings (ROSSIFER, 1960). Extracts of banana, pineapple, papaya, fig, mango, and Chinese gooseberries were individually added to Knudson's medium to raise *Phalaenopsis* seedlings. Banana extract was the most effective, followed by pineapple, papaya etc. in the order given above (ERNST *et al.*, 1970). In other studies, fractionated banana extract supported better growth of *Cattleya aurantiaca* seedlings than the whole extract. Seedlings 89–140 days old were used to determine the general growth index (ERNST, 1967a; ARDITTI, 1968). Seedlings with maximum number of roots, developed in water soluble and ethyl alcohol-insoluble fractions of the extract. Tomato juice was found to be very effective both for seed germination and seedling development of *Vanda* Joaquim (RAO and AVADHANI, 1964; ONG, 1969). Synergistic effects of banana extract, coconut milk, tomato juice and NAA or IBA on seed germination and protocorm development were determined in *Cattleya*, *Dendrobium*, *Phalaenopsis* and *Vanda* (PAGES, 1971; VALMAYOR, 1974). In most of the studies, sucrose was used as a carbohydrate source in the medium.

Studies undertaken in various laboratories emphasized the importance of the type and concentration of sugars used to promote both germination and growth of seedlings (LIDDELL, 1953; KARASAWA, 1966; ANDERSON, 1967; ARDITTI, 1967a; ERNST, 1967b; ARDITTI *et al.*, 1972). Seed germination and seedling development of certain terrestrial and epiphytic orchids improved with the addition of coconut milk to the medium (HEGARTY, 1955; LAWRENCE and ARDITTI, 1964; MCINTYRE *et al.*, 1974). However, coconut milk inhibited root and leaf production of *Dendrobium* seedlings (KOTOMORI and MURASHIGE, 1965), and induced abnormal proliferations in *Phalaenopsis* and *Vanda* seedlings (RAO and AVADHANI, 1964; ERNST *et al.*, 1970).

Larger seedlings resulted when *Cymbidium* and *Cypripedium* seeds were soaked for more than five hours and inoculated in completely sealed tubes (KANO, 1968). Prolonged soaking possibly leached out certain germination inhibitors present in the seed coat and the complete sealing of the tubes may have

increased the CO_2 level thereby promoting seedling development. *Cattleya* seedlings, developed in crowded condition showed poor root formation and due to limited or nonavailable nutrients required longer development time (ALBERTS, 1953; NORTHEN, 1962).

Streptomycin, terramycin (1–100 mg/l) and aureomycin inhibited *Cattleya* seedling growth (SHAW, 1953; O'NEILL, 1959), while in certain other plants (e.g. rose) antibiotics acted synergistically, enhancing the effect of foliar nutrients (NICKELL, 1952a, b; JORGENSEN, 1954, 1955).

The occurrence of vitamins in seeds has been recorded and during seed germination, vitamin C is formed in the cotyledons. Vitamins also play an important role in seed germination and seedling development (BONNER and AXTMAN, 1937; BONNER and BONNER, 1938). The positive growth effects of vitamins in orchids were reported in *Cattleya, Cymbidium* and *Laeliocattleya* (NOGGLE and WYND, 1943; WITHNER, 1959; LAWRENCE and ARDITTI, 1964; ARDITTI and BILS, 1965; ARDITTI, 1966a, 1967a, b; UEDA and TORIKATA, 1969a, b, 1972).

Under the influence of gibberellic acid the growth of *Cattleya* seedling improved 3–4 times in comparison with the control group but germination percentage was poor (HIRSCH, 1959).

To test their effectiveness, bark and peat extracts were incorporated into the medium. Seed germination and seedling development improved but certain barks (e.g. incense cedar and sugar pine) promoted better growth than others (FREI, 1973; FREI *et al.*, 1975).

The fresh weight of *Paphiopedilum* seedlings tripled when vegetable charcoal was added to Thomale GD agar medium and the addition of banana extract improved growth even further (ERNST, 1974). *Phalaenopsis* seedlings grown on similar media showed considerable weight increase. *Phalaenopsis amboinensis* seedlings supported by glass wool or cotton wool and grown on Knudson's C liquid medium, showed higher fresh weight than those grown on control agar medium. The fresh weight doubled when pyrex glass wool was utilized, as against cotton wool (ERNST, 1975).

3.6 Tissue Culture and Species Conservation

In many parts of the world, forests are making way for agricultural land, and in the process trees are being cut down along with their epiphytic flora (MILLAR, 1971; OSPINA, 1971). In such situations, elimination of ground orchids is also imminent. Very few countries are really concerned with the conservation of forests or of saving the individual species therein, more especially in the Asian countries. To save the orchid species from extinction several workers have used tissue culture techniques to raise plants by growing them in nurseries (HEY and HEY, 1966). As part of the conservation program hybridization work and agar media growth of endangered tropical species, has been extended (ALPHONSO, 1966, 1975).

4. Meristem Culture

The credit for the initiation of meristem culture technique goes to the late Dr. G. MOREL of INRA, Versailles, France (MOREL, 1960). Prior to the meristem culture of orchids, very successful results had already been obtained in carnations, dahlias, and potatoes (MOREL and MARTIN, 1952, 1955). *Cymbidium* seeds and seedlings were freed from the systemic mosaic virus that infected various parts of mature plants such as roots, bulbs, leaves, and flowers. It was believed that the young meristematic regions of plants were either not infected by the virus or the tissues had a defence mechanism to ward off the pathogen (JENSEN, 1955). Based on this assumption virus-free clones of *Cymbidium* plants were established by using the axillary buds as inoculae. In addition to obtaining the virus-free clones, this technique also opened another important avenue for plant multiplication and for obtaining a considerable number of seedlings of valuable commercial orchids in a relatively short period of time.

In a lecture delivered to the *Cymbidium* Society, MOREL (1965) discussed various aspects of meristem culture in detail. *Cymbidium* vegetative propagation by bulbs is a slow process, requiring an average of 10 years to obtain good-sized plants. The prospects of obtaining good plants from seeds are very low due to heterozygosity of hybrids. Green bulbs are the source materials for buds in *Cymbidium*, each bud being processed into an explant with sizes varying from 0.5–1 mm with 3–4 leaf primordia. Even on a simple mineral medium like Knop's each explant regenerates and gives rise to 2–3 protocorms. The cut portions of protocorms grew well on high salt medium supplemented with coconut milk, or banana extract. Each protocorm was further divided into 4–8 pieces and within one month each piece regenerated into a full protocorm; the uncut or undamaged protocorms developed into plantlets.

Cattleya explants were also obtained from buds. Growth of explants, the recommended size of which is around 2.4 mm, was much better in liquid than on agar medium, and from each inoculum some 12 protocorms were formed. Amino acids, a good source of nitrogen, as found in peptone, IAA or NAA (1 mg/l) were beneficial. Successful results were also obtained in *Dendrobium*, *Lycaste*, *Miltonia*, *Odontoglossum*, and *Phaius*. It was suggested (MOREL, 1965) that by using such techniques large-scale orchid plant production could be standardized, as in roses or carnations. Production of large numbers of flowers of good quality and color and for the required period in the year could become feasible. Thus the application of this technique along with hybridization programs, could be very beneficial to orchid growers. Commercial implications such as standardizing the orchid varieties, production of large numbers of flowers of good quality and the introduction of new hybrids into the market, were discussed (MOREL, 1965; BLOWERS, 1966, 1967).

The process and points of regeneration from the inoculae in *Cymbidium* and *Cattleya* were different. New *Cymbidium* meristems differentiated sub-apically, whereas in *Cattleya* the wound tissue near the leaf base proliferated and gave rise to meristematic regions (CHAMPAGNAT *et al.*, 1966; CHAMPAGNAT and MOREL,

1969; MOREL, 1971). However, perpetual proliferation of protocorms in both was achieved by using segmented tissues of the protocorm. The pattern of protocorm production in *Miltonia* and *Odontoglossum*, was similar to that of *Cattleya*. In *Vanda*, proliferation of cortical tissues near the apical region of the bud was common, some of which produced protocorms. In all such experiments MURASHIGE and SKOOG's medium (1962) was more effective than Knudson's medium. It is important to note here that the apex of the axillary bud did not participate in regeneration; only the meristematic tissue in the basal region of the bud proliferated and gave rise to new structures. Protocorm formation required less time and was more sporadic in *Cymbidium*, than in the other orchid genera tested (MOREL, 1971). Meristem culture of *Miltonia* resulted in great proliferation with as many as a hundred plants formed from a single bud (MOREL, 1964). Thus in papers published from 1960–1971 the foundations for meristem propagation work were well laid—MOREL'S success in *Cymbidium* was a significant achievement in this direction.

The benefits of meristem culture attracted the attention of many researchers and work on various valuable orchid hybrids was initiated in different laboratories. The results obtained with regard to growth and proliferation of tissues in response to diverse media and growth adjuncts used, were recorded. Details were discussed regarding the nature of propagation material, method of obtaining sterile meristem, size of explant, pH of the medium and different environmental conditions suitable for cultures (BERTSCH, 1966; MARSTON, 1966, 1967, 1969; MARSTON and VORAURAI, 1967; GOH, 1971). The techniques and details involved in meristem tissue propagation in vitro were explained by ISLEY (1965) and RUSSON (1965) in simple terms for the benefit of orchid growers. The application of meristem culture either to eliminate virus infection in clonal plants or large-scale production of asexual seedlings and other horticultural benefits, have been discussed in many different publications (ISLEY, 1965; RUSSON, 1965; JESSEL, 1966; GRIPP, 1966; BLOWERS, 1966, 1967; JASPER, 1966; LINDEMANN, 1967; VORAURAI, 1968; MARSTON, 1969; LINDEMANN *et al.*, 1970; BEALLE, 1971; BILTON, 1971; MCLELLAN, 1971; TAYLOR, 1971; THOMPSON, 1971; see also Chap. I.2 of this Vol.). The commercial implications and registration problems in obtaining patents for mericlones have also been analyzed (LECOUFLE, 1971; HUMPHREYS, 1971).

The successful results achieved in different orchid genera by using various plant parts as inoculae are briefly summarized in the following paragraphs. Other factors that promoted the growth of such cultures and the time involved to obtain suitable plantlets are emphasized. Unless mentioned otherwise, only the hybrid plants were made use of by different workers (Table 1).

4.1 Main Apex and Apices from Buds

Axillary buds from pseudobulbs of *Cattleya*, first cultured in liquid medium, were transferred to agar medium after 2–5 weeks and showed increase in size in 4–10 weeks. The axillary buds were subdivided into smaller units and inoculated in fresh medium. Two months after sub-culturing, small plantlets, which could be transferred to greenhouse pots, were formed. Coconut milk promoted tissue

growth and seedling development (SCULLY, 1967). RUTKOWSKI (1971) observed that plantlet development could be completed without the intervening protocorm stage in *Cattleya* cultures.

Cymbidium shoot tips were cut either vertically or transversely into sectors, cultured in liquid medium for a month, and then transferred to an agar medium. The transversely cut sectors developed into protocorm-like bodies (WILFRET, 1966). Similar results were obtained by SAGAWA *et al.* (1966) in six-week-old cultures. In *Cymbidium talma* a longer period of 18 months was required. In a series of papers by UEDA and TORIKATA (1968, 1969a, b, 1970a, b, 1972) the effects of different growth substances on meristem cultures of *Cymbidiums* were outlined. Arginine, aspartic acid, Kinetin, NAA, and yeast extract supported the growth of callus and different organs. The relative merits of concentrations and combinations of these growth substances were determined. In cultures maintained in dark conditions, NAA below 0.1 mg/l promoted shoot formation, and root formation was abundant at 0.6 mg/l.

By application of free cell culture technique, plantlets were obtained from *Cymbidium* hybrids (STEWARD and MAPES, 1971). Shoot apex, when grown on agar medium, supplemented with coconut milk and NAA, developed callus which was transferred to a liquid medium of the same composition. Free cell production was better when NAA was replaced by 2,4-dichlorophenoxyacetic acid, (2,4-D). Creamy white protocorms were formed and on transference to agar medium, developed into plantlets within 9 months (STEWARD and MAPES, 1971). RUTKOWSKI (1971) recorded that in *Cymbidium* explants, peripheral tissues, mostly epidermal and sub-epidermal in origin, grew well and developed into plantlets, and that repeated sub-culturing and multiplication of plantlets was possible.

Buds excised from *Dendrobium* bulbs, as well as those from leaf axils, grown on Vacin and Went's liquid medium supplemented with 15% coconut milk showed protocorm like bodies in 4–5 weeks, and plantlets in 8 weeks (KIM *et al.*, 1970). Proliferation was common, and when separated, they developed into regular plants. Axillary buds from shoots grew better than those from bulbs. Occasionally the intervening protocorm stage was absent. In some *Dendrobium* cultures, a 2-year period was recorded for plantlet formation and flowering (SAGAWA and SHOJI, 1967).

Excised apices of *Rhyncostylis gigantea* were cultured on a composite agar medium supplemented with NAA and coconut milk. In 3½ months plantlets developed and in seven months, after the fourth sub-culturing, callus was formed. The callus also differentiated into plantlets upon subsequent transfer to fresh medium (VAJRABHAYA and VAJRABHAYA, 1970).

In Hawaii, KUNISAKI *et al.* (1972) established meristem cultures of *Calanthe, Cattleya, Dendrobium, Miltonia, Odontonia, Odontoglossum, Phaius, Rhyncostylis* and *Vuylertekeara. Phalaenopsis* did not respond to the usual technique, and certain modifications were necessary with *Vanda.* In *Vanda* Miss Joaquim and three other hybrids, axillary buds were inoculated on agar medium supplemented with 15% coconut milk. Eight weeks later the swollen buds were transferred to a liquid medium. In 10–12 weeks the explants proliferated, and when transferred to a 1–2% sucrose-agar medium, developed into plantlets. Omission of sucrose in the medium, increased proliferations (KUNISAKI *et al.*, 1972).

Meristem cultures were also used for induction of polyploidy in *Cymbidium*, *Dendrobium* and *Vanda* hybrids. The cultures were treated with colchicine (0.1–0.5%) and the tetraploid and hexaploid plantlets that resulted were grown to maturity. Chromosome numbers were determined by squash preparations (WIMBER, 1963; WIMBER and VAN COLT, 1966; VAJRABHAYA and VAJRABHAYA, 1970; SANGUTHAI *et al.*, 1973; SANGUTHAI and SAGAWA, 1973; VAJRABHAYA, 1975).

GOH (1973) used both apical meristems and axillary buds of *Aranda* cv. Deborah, a monopodial orchid, as inoculae on liquid and solid medium. Plantlets developed directly from the former without protocorm formation. On further growth and regeneration, tissues from axillary buds developed into plantlets in 8–9 months. In *Aranda* hybrid, callus formation from axillary buds was spontaneous when grown in Vacin and Went's liquid medium supplemented with 20% coconut milk. The callus could be maintained by sub-culturing to a fresh medium without coconut milk. Thus coconut milk was necessary only for initiation of callus and not for further growth (LOH, 1975). If the callus was left in the same medium for a longer time, differentiation set in, leading to formation of plantlets (Figs. 12, 13). Considerable variation was noticed between the tissues formed in liquid and solid media of the same composition (Figs. 14, 15; LOH, 1975). Plantlets were formed in about 90 days from shoot tips of *Vanda* hybrid, a monopodial orchid. Sucrose was not necessary at the differentiation stage although coconut milk was used throughout the experiment (TEO *et al.*, 1973). In similar studies successful results were obtained in other tropical orchid hybrids (TEO and TEO, 1974a, b). Phenotypic variations were found within the clones of *Dendrobium* propagated by the meristem culture (VAJRABHAYA and VAJRABHAYA, 1974).

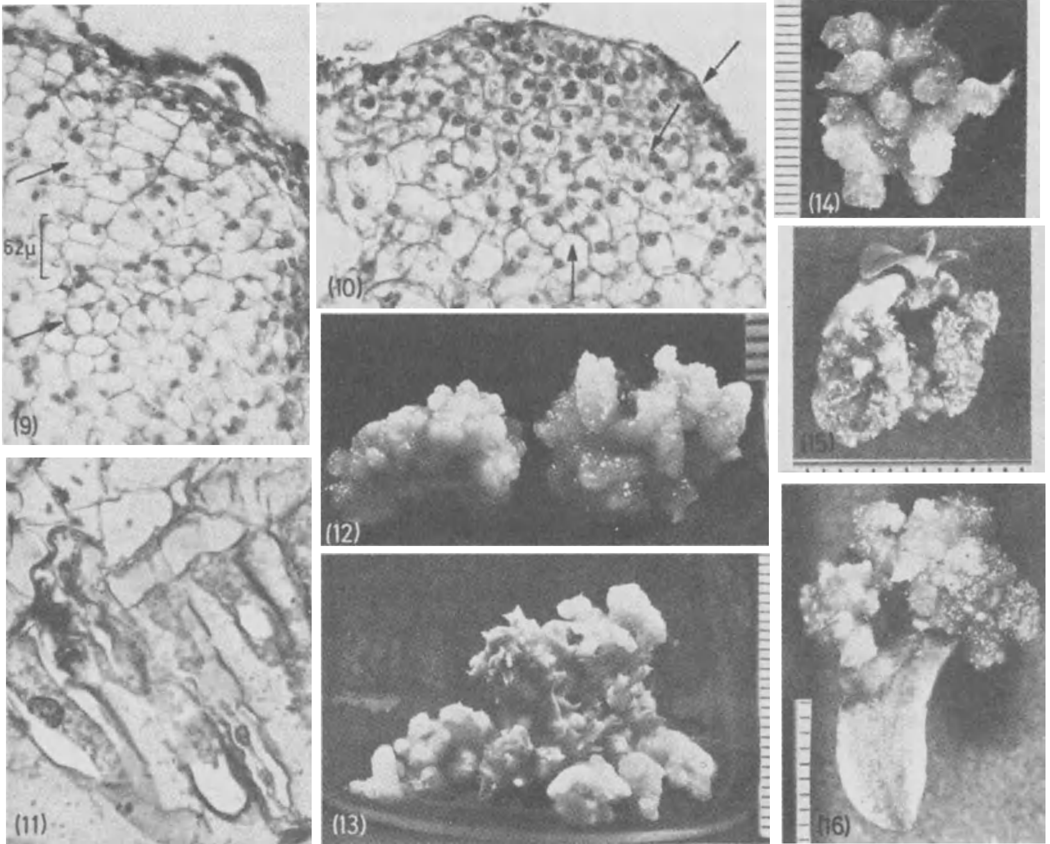
4.2 Nodal Regions from Stems

Other methods not having proved successful, stem segments of *Phalaenopsis* (3–4.5 cm long) with a node were used to start the cultures (SAGAWA, 1961; SCULLY, 1965, 1966). Buds enlarged in two weeks followed by leaf and root development in 6–20 weeks. 30 weeks old plantlets ready for transfer had from 4–5 leaves and 1–2 roots. Plantlets developed in *Vanda* Miss Joaquim in 2–3 months when a similar technique was used (SAGAWA and SEHGAL, 1967). In this type of study, bud, which otherwise would remain dormant, is induced to grow under culture conditions to obtain new plantlets.

4.3 Leaves and Leaf Tips

Various methods of regeneration are recorded in certain orchids, but considering the size of the family only a few species or hybrids have been studied with a view to analyze their regenerative potential (HOLTTUM, 1953; CAMPBELL, 1964; HOLM, 1965; TAYLOR, 1967; KUMAR, 1973). Moreover, only very few orchids have been used in tissue culture work to raise seedlings from different vegetative parts. In vitro culture studies on leaf or leaf tip regeneration are summarized here.

CHAMPAGNAT *et al.* (1970) used seedling leaves of *Cattleya* hybrid to induce further growth. Axillary meristem at leaf base gave rise to a callus that differen-



Figs. 9–16. *Vanda* Joaquim. Figs. 9, 10. L.S. callus showing early and late stages in the development of superficial meristem, arrows indicate regions of cell division. Fig. 11. Enlarged view of unicellular rhizoids. Figs. 12–15. *Aranda* Deborah. Figs. 12, 13. Early (12 weeks) and later (20 weeks) stages of callus development from apices grown in Vacin and Went liquid medium + 20% coconut milk. Figs. 14, 15. Apices grown on liquid and solid media, 20 weeks, note protocorm development in the former, mostly callus and a few seedlings in the latter. Fig. 16. *Aranda* Nancy. Callus and protocorm development from a seedlings leaf on Vacin and Went liquid medium, 14 weeks old. (mm scale)

tiated into many protocorms. IAA, kinetin, casein hydrolysate, liver extract, and vitamins induced variable growth. The growth potential of seedling leaves was demonstrated and they could thus be used to obtain plantlets in great numbers. Young leaf tips of *Dendrobium*, *Epidendrum* and *Laeliocattleya* were used by CHURCHILL *et al.* (1970, 1971) to induce regeneration. After 6–7 weeks small calli and protocorms developed, and in 10 weeks small plantlets were formed. On separation each grew into a normal plant. The importance of using both liquid and solid media, and appropriate times for transferring tissues from one medium to the other were subsequently discussed (CHURCHILL *et al.*, 1973). Under the influence of orthochlorophenoxyacetic acid, callus was obtained from *Epidendrum* leaf tips and at optimal concentration (2 mg/l) growth improved, as

evidenced by an increase in the dry weight (RUDOLPH *et al.*, 1972). Under the influence of coconut milk in the medium, leaf tips of *Aranda* hybrid gave rise to callus (Fig. 16) and within four months plantlet development was complete (LOH *et al.*, 1975). Two types of leaves (succulent and non-succulent) were formed in this hybrid, the former showing greater growth potential. All the studies mentioned above clearly demonstrate the growth potential of leaves and young leaf tips. It is probable that other commercially important orchid hybrids may show similar growth potential, and studies of this kind may prove helpful to increase the production of desired clonal plants. Further, by using leaves as explants, the damage to the clonal plant can be minimized or avoided.

4.4 Inflorescence and Buds from Inflorescence

Most orchids produce racemose inflorescences and, generally buds in the basal region of the axis remain dormant. After removing bracts, inflorescence primordia (1.5 cm long) of *Ascofinetia*, *Neostylis*, and *Vascostylis* were inoculated on liquid medium. In a few weeks abundant proliferations were formed from hypodermal tissues of the axis. Tissues were transferred into agar medium after four months and seedlings ready for transplanting developed in 15 months. Sugar was required only in the initial stages and the addition of coconut milk, potato or banana extract, improved growth. When primordia from larger inflorescences were used (3 cm or more), the axis elongation was evident and buds developed into small flowers (INTUWONG and SAGAWA, 1973). By a similar technique INTUWONG *et al.*, 1972; INTUWONG and SAGAWA, 1974), plantlets were obtained within five months in *Dendrobium*, *Phalaenopsis* and *Vanda* (SINGH and SAGAWA, 1972; SINGH, personal communication).

ROTOR (1949) observed that flower stalks with buds, obtained from the middle portion of an inflorescence, served as useful material in *Phalaenopsis*. Plantlets with few leaves and that 1–2 roots developed in five months. Buds at basal or terminal nodes developed into flowers and were therefore discarded. In a similar study, TSE *et al.* (1971) recorded poor growth in *Phalaenopsis* as compared with *Cattleya* or *Cymbidium*. Certain surgical techniques were used before inoculating i.e. (1) $^2/_3$ of the bud was removed; (2) the bud was cut vertically, or (3) the bud was punctured longitudinally from apex to base. Two weeks later the intact bud developed into a shoot primordium whereas those injured showed signs of callus growth. On further growth from each callus mass 3–4 plantlets developed within 10 weeks. These were separated and grown as individual plants. Production of a greater number of plants was thus possible by using the above technique. Murashige and Skoog's medium with NAA was more effective than Knudson's medium (TSE *et al.*, 1971).

4.5 Rhizome and Roots

A suggestion was made by BEECHEY (1970b) that orchid aerial roots could be used for in vitro propagation of plants as done in dicots and other monocots. If the attempts were to be successful it would open a tremendous opportunity, compa-

rable to meristem culture for growing useful orchid hybrids without sacrificing either the main apex or lateral buds of mother plants. Rhizome tips of *Cymbidium goeringii* (= *Cymbidium insigne*) and *Cymbidium pumilum* inoculated on agar medium supplemented with kinetin (10 mg/l) and maintained in the dark gave rise to masses of rhizomes without shoot formation but when L-arginine (10^{-3}M) was added, shoots developed (UEDA and TORIKATA, 1972). *Epidendrum* root segments grew into thin, etiolated, elongated structures without producing any calli or plantlets (CHURCHILL *et al.*, 1972). The medium recommended for wheat root tips by OJIMA and FUJIWARA (1962) was used; a similar type of root growth was recorded in the case of *Vanda* Miss Joaquim (GOH, 1970b).

In many of the studies mentioned in this section callus development was common. The developmental changes that lead to organogenesis and plantlet formation followed the events outlined in Figure 8.

Induction of mutagenesis is an important technique to improve the quality of plants. HARN (1968) outlined the advantages of mutagenic in vitro culture technique to achieve greater variety of form and growth in orchid hybrids. Importance of ploidy and nuclear behavior in inducing the structural alterations, as demonstrated in other flowering plants, were mentioned. Proliferated tissues resulting from either embryo or protocorm cultures could be subjected to the influence of irradiation and mutagenic chemicals and redifferentiation into better quality plants (NAIL, 1966; HARN, 1968; CHANGYAWL, 1970; SAULEDA, 1971).

The importance of choosing a suitable medium to obtain successful results of embryo culture can not be overemphasized. Earlier workers paid considerable attention to either reducing or increasing the mineral contents of a defined medium which promoted better growth of a particular orchid tissue (HELLER, 1953; WITHNER, 1959; RAGHAVAN and TORREY, 1964; REINERT and MOHR, 1967; ZIEGLER *et al.*, 1967). When the author worked at Versailles with the late Dr. Morel, specific mineral requirements of different orchids were determined (MOREL and RAO, unpublished data). Similarly, the inclusion of different growth adjuncts and vitamins in the medium resulted in considerable improvement in tissue growth (WITHNER, 1955, 1959). Many workers pointed out the relative merits of such substances; for example, coconut milk (KIM *et al.*, 1970; KUNISAKI *et al.*, 1972; INTUWONG and SAGAWA, 1973), auxins (BOESMANN, 1962), cytokinins (REINERT and MOHR, 1967; KANO, 1971; FONNESBECH, 1972; PIERIK and STEEGMANS, 1972), and gibberellins (LINDEMANN, 1967).

5. Commercial Aspects of Orchid Industry

Orchids are commercially grown in many countries (see also Chap. I.2 of this Vol.). The cut flower trade includes the hybrids of the following genera: *Arachnis* (Scorpion orchids), *Cattleya*, *Cymbidium*, *Dendrobium*, *Epidendrum*, *Oncidium*, *Phalaenopsis*, *Renanthera*, and *Vanda* (PURSEGLOVE, 1972). The other genera are no doubt important but their role in trade is a minor one. Monopodial orchids are propagated by stem cuttings with an intact growing apex, a few leaves and young roots. In sympodial orchids, propagation is by

separation of pseudobulbs (HENDERSON and ADDISON, 1956). Considerable effort, management, and controlled conditions in greenhouses are essential to grow orchids in temperate countries. In the tropics the humidity, rainfall and temperature provide suitable conditions for growing a wide variety in the open, so that both terrestrial and epiphytic orchids grow luxuriously throughout the year, producing a continuous supply of flowers for the market and in several countries of Southeast Asia this facility has been fully utilized for promoting the orchid trade. A general survey can be attempted on the following lines: extent of orchid industry in different countries; economics involved in terms of total annual consumption of flowers or plants; and export and import tendencies. The details are not easy to obtain because there are no definite statistical figures available for most countries, and if present, they are difficult to procure. No doubt many well-known orchid-growing companies, especially in the USA, Australia and France, maintain their own records, but the data they possess are generally not intended for public information; however, such information as is available is summarized below.

The officials of the Australian Orchid Exporters Association kindly provided the following data with regard to the orchid industry in that country. Large concerns located on the perimeter of Sydney produce most of the cut flowers, chiefly *Cymbidium* and *Cattleya*, and these are exported mostly to the USA. It is estimated that nearly one million blooms are exported each year and the average wholesale price for each bloom varies from US 50 cents to $ 1.50. Almost an equal quantity of flowers is sold in local markets. Many growers and companies employ tissue culture techniques for mass production of high-yielding populations and both seeds and meristems are used (BRYANT, personal communication).

The main commercial orchid-growing areas in England are Cooksbridge, Slough, Kingsteignton, Leeds, and Tunbridge Wells. Apical meristem culture is employed commonly in many gardens and laboratories to obtain the desired clones of *Cymbidium* hybrids. Plants and flowers thus produced are meant for local consumption (HUMPHREYS, personal communication). The economics involved are discussed by HOLDGATE (Chap. I.2 of this Vol.).

In France, important orchid-growing areas are in Boissy St. Leger, Angers, Antibes, Cote d'Azur, Brittany and Paris, in that order. The total estimated area of the green houses is around 6 hectares. It is estimated that the annual value of plants produced ranges from US$ 800000 to 1 million and that of flowers is around 0.5–0.75 million US$. Official and authoritative figures for trade and commerce may be variable and the information given above is supplied by one of the professional growers and another friend working in the Agriculture Research Institute. Plantlets obtained by means of tissue culture are exported, and imported orchids come mainly from Singapore, Malaysia, and Thailand through German and Dutch channels (VACHEROT personal communication). It is well known that organizations like Vacherot and Lecoufle in France were the first to adopt the meristem culture techniques for mass production of clonal plants. Similarly seeds or stud plants were acquired or sent from other countries to France and these were used as source materials for the propagation of valuable orchids and the plants thus produced were exported.

In Indonesia, the use of tissue culture techniques to speed up the propagation of desirable clones has begun, but since the mature plant parts are necessary for propagation there has been drop in flower production. Tissue culture may well become useful to compensate this loss (SASTRAPRADJA, 1975). In 1972 about 35064 kg of orchids (cut flowers) were exported from Indonesia to Singapore, Hong Kong, Japan, various European countries, the USA and Australia. In 1974 exports increased to 46017 kg but the actual financial value involved is not given (YASNI, 1975).

The establishment of an orchid industry in Papua, New Guinea has begun, and there, tissue and embryo culture methods are being employed to increase the number of native species and hybrids (MILLAR, personal communication).

From New Zealand, cut flowers of *Cymbidium* are exported mainly to Europe and the United States and the annual value of export trade is about US$ 180000. Most of the orchids are grown in the warmer parts of the North Island (GILES, personal communication), and very few orchids other than *Cymbidium* are grown commercially because of the heating requirements.

There are only two orchid nurseries in all of Norway and the annual estimated value of orchid trade is around US$ 400000. Nearly 50% of the orchids are grown in Norway and the rest are imported (BRAGDO-AAS, personal communication).

In Portugal, orchid cultivation has long been traditional at certain places but growing flowers for the cut flower trade is a recent business in the country. Orchids are mainly grown on the island of Madeira, in the Atlantic Ocean, a few miles southwest of Lisbon. The island is of volcanic origin with mountains reaching almost 6000 ft. The climate is favorable for subtropical, temperate and even alpine orchids. *Paphiopedilum* and *Cymbidium* hybrids are the important genera under cultivation. The cut flowers are mainly exported to Germany, Switzerland, and Belgium. The annual value of flowers exported in 1974 was around US$ 120000; 66% of which involved *Paphiopedilum* and 34% of *Cymbidium*. Meristem culture techniques are employed for mass production of high-quality plants. A vigorous breeding program is also being conducted for *Cattleya*, *Cymbidium*, *Paphiopedilum* and a few other genera (GALANT, personal communication).

The history of the orchid industry in Singapore, including trade and export has been handled by BURKILL (1964) and EDE (1965). In 1950 the annual export value was US$ 4000. Most of the orchids from Singapore are exported to the European continent and the United Kingdom. The annual value of export trade in 1974 exceeded 5 million Singapore dollars (US$ 2.3 million; ALPHONSO, 1975). New orchid farms, some of them as large as 250 acres, are being established in South Johore and other parts of West Malaysia. Interest is also developing in the sale of cuttings of monopodial orchids like *Arachnis* Maggie Oei and other well-known hybrids to local and outside growers (YEOH, 1975). While discussing the necessity for careful selection of clonal plants to establish the orchid farms some of the difficulties experienced and disappointing results obtained by the growers have been pointed out. Out of ignorance either virus-infected clonal plants or plants suffering from bud drop deficiencies were planted in many parts of West Malaysia. Good clonal material is in short supply. Further, the conventional method of propagation is slow to obtain plants of desirable clones. Therefore the

usage of tissue culture or meristem culture techniques is recommended to obtain a large number of quality plants (TEO, 1975).

The orchid industry in South Africa, at present, is small but increasing interest in its development is shown by many growers. The use of tissue culture methods for propagation of plants has begun and several commercial growers are selling plants from flask cultures and mericlone cultures (STEWART, personal communication). Details are published in the South African Orchid Journal from time to time.

A cooperative organization has been started with government support, in Sri Lanka, to establish an export-oriented orchid industry. About a million plants were bought from Singapore and Thailand to establish nurseries and the cut flower industry (WEERASEKARA, 1975).

The annual value of imported orchids to Sweden is around US$ 1 million, coming from Holland, Southern France, and Burma (WIKESJO, personal communication).

Tropical and subtropical climates prevail in Taiwan and the cultivation of orchids is increasing. Many of the growers are using meristem culture techniques for propagation of well-known hybrids and for the induction of polyploidy. Cattleyas are the most common orchids in Taiwan, used both for export and domestic consumption. Others such as *Cymbidium*, *Dendrobium*, *Phalaenopsis* and *Vanda* are grown on a less extensive scale. Tissue culture and meristem culture techniques have been used for mass production of seedlings of the above genera (CHEN, 1975).

In Thailand, most of the places where orchids are grown for commercial purposes are within a 100-km area around Bangkok. The annual export value is estimated at US$ 6.5 million. A very small proportion of the flowers produced are used locally. At least 10 large and numerous small laboratories raise orchid seedlings by the tissue culture method, from the pots or seeds supplied by the breeder. Seedlings grown and ready to be transferred to community pots are sold for US 5 cents a plantlet. Likewise some 200000–400000 plantlets are exported annually to other countries at a cost of US 25 cents a plantlet. Plantlets are grown by horticulturists for a considerable time in their nurseries to make sure that they are disease-free (VAJRABHAYA, personal communication), before they are exported.

In the United States, orchids are grown on a large scale in Hawaii, California (particularly Santa Barbara, the San Francisco Bay area, San Diego, and Los Angeles), Southern Florida, and in or near urban areas such as New York, Philadelphia, Chicago, St. Louis, Dallas–Ft. Worth and certain other areas in the states of Illinois and Washington. Most of the big orchid nurseries and companies derive their plants by hybridizing and growing them under aseptic culture, including meristem cultures. Modest estimates indicate that the annual value of the cut flower industry in the Santa Barbara area is approximately US$ 2 million, in all the regions around California $ 10 million, and the total annual value of cut flower consumption is approximately 50–60 million. Very little is exported. Orchid flowers can sometimes be very expensive—US$ 1.50–3.00 each—depending on the occasion and the demand (HETHERINGTON, 1971). The financial implications involved in promoting the breeding program and sales of orchid plants are discussed by SCULLY (1971).

From the above account it is very clear that the application of tissue culture techniques has rendered valuable results in the case of orchids. Commendable results have been obtained in the recovery of disease-free clones, preservation of valuable germ plasm and rapid clonal multiplication of selected varieties (MURASHIGE, 1974). The significance of these results in the promotion of trade and industry is no small one.

By the successful results obtained from meristem culturing, some of the best orchids may become commonly available and within the price range of more people. Possession of plants with such beautiful flowers may spread further interest to take up orchid cultivation with enthusiasm (BERTSCH, 1966).

Many other possibilities exist for future research, e.g. inducing mutations in the tissue culture of orchids may be helpful to obtain plants of greater commercial value. The possibilities of inducing polyploids have also been looked into in certain orchids. The recent studies made on the growth potentialities of seedling leaves of *Aranda, Epidendrum* and *Laeliocattleya* are very significant and similar researches, if conducted in other orchids, may yield beneficial results. As has been well known for the last few years, protoplast culture (see BAJAJ, 1974) has opened another important field of research and this method of culture work may become helpful in producing new varieties or species of orchids. Where meristem or embryo culture is a failure the protoplast culture may yield beneficial dividends.

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References see page 207.

4. Tissue Culture in the Citrus Industry

J. BUTTON and J. KOCHBA

1. Introduction

Species of the genus *Citrus* are all believed to be native to the tropical and subtropical regions of Asia and the Malay Archipelago (WEBBER, 1967). From these regions the production of citrus fruits has spread to all tropical and subtropical zones in the world. Producing regions now occupy a belt extending round the globe between approximately 35° N and 35° S (BURKE, 1967).

By 1961–1962, there were 49 countries with commercial plantings covering a total of some 1.8 million ha. Since then the area has been increased and is still expanding, particularly in North Africa (ANON, 1974b). A marginal overproduction is expected by 1980. The USA is by far the greatest citrus-producing country, accounting for about one-fourth of the world's production (Table 1). The balance is spread over numerous countries throughout the citrus-growing belt. Oranges and tangerines account for over 82% of the total citrus crop. Grapefruit contributes some 8.5%, while lemons and limes make up the balance (Table 2). There is a definite swing in consumer preference to easily peeled varieties (ANON, 1974b). In order to satisfy this shift in demand, much research is now being carried out on the breeding and selection of new, suitable cultivars.

Table 1. The main citrus producing countries of the world[a]

Country	Total production (metric tons × 1000)	
	1971 (% of total)	Projection (% of total) 1980
USA	10788 (28.5)	13500 (23.9)
Brazil	3522 (9.3)	5610 (9.9)
Japan	2938 (7.8)	5000 (8.8)
Italy	2349 (6.2)	2902 (5.1)
Spain	2118 (5.6)	5610 (9.9)
Mexico	1775 (4.7)	2315 (4.1)
Argentina	1542 (4.0)	1950 (3.4)
Israel	1542 (4.0)	1650 (2.9)
India	1370 (3.6)	2096 (3.7)
China	908 (2.4)	1220 (2.2)
Moroco	771 (2.0)	1570 (2.8)
Egypt	732 (1.9)	1729 (3.1)
Other countries	7546 (19.9)	11399 (20.2)
World total	37901 (100)	56551 (100)

[a] Data extracted from FAO. Bull. CPP: CI 74/2.

Table 2. Commercial varieties of citrus fruits[a]

Fruit Type	Estimated world production (metric tons × 1000)	
	1975 (% of total)	1980 (% of total)
Oranges and tangerines	34000 (82.1)	46870 (82.9)
Lemons and limes	3880 (9.4)	4960 (8.8)
Grapefruit	3560 (8.5)	4720 (8.3)
Total	41440 (100.0)	56550 (100.0)

[a] Data extracted from FAO. Bull. CPP: CI 74/2.

Since more than 50% of the world citrus crop is consumed fresh (ANON, 1974b), the internal quality and the external attractiveness is of importance, particularly in the face of overproduction. Escalating costs further complicate the producer's position. Constant efforts are being made to increase yields per unit area and to improve the external appearance and internal quality of fruit. These efforts are divisible into a number of interdependent scientific disciplines. Breeding and selection of new scion and rootstock varieties is being undertaken in many countries. The main objectives of this work are not only to increase yields and improve quality, but to select cultivars resistant to pests and diseases. These aims immediately involve the cooperation of pathologists and entomologists. Physiologists, soil scientists, and horticulturists are also involved in both basic and applied aspects. Workers in all fields of research on citrus are faced with problems of seasonal changes and availability of research material. The lack of control over interacting environmental factors further complicates the task of the research worker. It is in overcoming these common problems that the technique of tissue culture can contribute significantly to the advancement of the citrus industry.

In this article the authors have attempted to outline the problems involved in many aspects of research connected with the citrus industry. Particular emphasis has been placed on the past, present, and future role of tissue culture as a tool in the citrus industry.

2. Polyembryony and Commercial Citrus Propagation

In the commercial culture of tree crops, uniformity within the orchard is an important objective. Since fruit tree scions are invariably grafted onto rootstocks, tree uniformity is dependent upon both scion and rootstock. The selection of suitable scion material poses no serious problem to the experienced nurseryman. In many genera of fruit trees however, the lack of uniformity in seedlings used as rootstocks poses serious problems (HARTMANN and KESTER, 1975). Fortunately this problem does not arise in *Citrus* as only highly-polyembryonic clones are selected for the provision of uniform rootstock seedlings.

Most *Citrus* species are polyembryonic and produce from one to 40 adventive embryos in the nucellus (FURUSATO *et al.*, 1957). Polyembryony is believed to be a

recessive, hereditary character controlled by a series of multiple genes (MAHESHWARI and RANGASWAMY, 1958). These genes may regulate the synthesis of a potent inhibitor of embryogenesis (ESAN, 1973) in nucellar cells of monoembryonic *Citrus* varieties. In polyembryonic varieties, the induction of nucellar embryos in vivo appears to be dependent upon a stimulus. This is provided by pollination, and possibly by fertilization and the early development of a zygotic embryo (FROST and SOOST, 1968). The extent of polyembryony is influenced by the nutritional status of the fruit (TRAUB, 1936), the pollen parent, and environmental factors (FROST and SOOST, 1968).

Theoretically all nucellar trees should be genetically identical to the mother tree (FROST and SOOST, 1968). In reality, however, phenotypic variability between nucellar seedlings can be quite marked, and in many cases is unlikely to be due only to juvenility. It is unlikely that "somatic fertilization" takes place in *Citrus* (MAHESHWARI and RANGASWAMY, 1958). However, the possibility cannot be excluded that pollination, and particularly fertilization, could influence genome expression in a nucellar embryo. The genetic inconsistency between nucellar embryos is further suggested by IGLESIAS *et al.* (1974). They found that peroxidase zymograms varied qualitatively between plants of the same cultivar and even between seedlings (presumably nucellar) derived from the same seed. The chimeral origin of certain *Citrus* clones could explain variation in nucellar offspring (BITTERS and MURASHIGE, 1967). Various proportions of nucellar cells may thus carry the mutation reflected in the phenotype of the mother tree.

The limited variability of nucellar seedlings poses no problem when they are used as rootstocks. When they are intended for the establishment of rejuvenated, disease-free "lines" of existing clones however, this variability is undesirable. The problem is aggravated by the fact that final selection of nucellar lines must be delayed until the bearing and fruit characteristics have been assessed. It is significant to note however, that the natural production of nucellar lines is restricted to seed-bearing, polyembryonic clones.

3. Morphological and Physiological Studies Involving Citrus Tissues

One of the most significant applications of tissue culture generally is in the study of isolated cells, tissues, or organs. Detailed observations can be made on differentiation, organogenesis, and growth, without the uncontrollable, confounding effects of surrounding tissues and the environment. Thus, the direct influence of selectively applied conditions on isolated systems can be established. Observations made in such systems often have far-reaching, practical applications.

3.1 Nutritional Requirements and Culture Conditions

Many nutrient media have been used in the culture of *Citrus* tissues. Pioneering work during the 1950s and early 1960s was usually carried out on WHITE'S (1943) medium with various modifications. Other media such as those of GAUTHERET

(1957), HELLER (1953), NITSCH (1951), and TUKEY (1933) were also used. Complex organic substances such as casein hydrolysate (CH), coconut milk (CM), yeast extract (YE), and orange juice (OJ), were frequently incorporated with various combinations and concentrations of growth regulators.

The success of these media was generally limited to the continued development of structures already initiated in vivo, and to the limited proliferation of unorganized tissue. It appears that limited somatic embryogenesis did occur in nucellar explants in which some natural nucellar embryos were already present. These immature embryos generally failed to develop into plantlets. Once the medium of MURASHIGE and SKOOG (1962) became available however, progress was stimulated greatly, particularly in respect to organogenesis and somatic embryogenesis.

3.1.1 Inorganic Constituents

The mineral elements of MURASHIGE and SKOOG (MS) are generally adequate, although probably not entirely optimal for all *Citrus* tissue cultures. This is in keeping with views that there is widespread consistency in inorganic requirements in spite of variability in cultured tissues (MURASHIGE, 1974). The main difference between the MS medium and other formulations is in the concentration and source of nitrogen. Most media contain only nitrate, while the MS contains a relatively high concentration of ammonium in addition to a higher nitrate level. It appears possible that embryogenesis in unorganized *Citrus* tissues may be enhanced by NH_4NO_3 as reported by REINERT and TAZAWA (1969) for *Daucus* cell cultures. The calcium concentration is also somewhat higher in the MS formulation than in others. There appears to be no necessity to modify the concentrations of inorganic constituents of the MS medium for the culture of *Citrus* tissues.

3.1.2 Carbohydrates

Sucrose satisfies the carbohydrate requirements of most tissue cultures (MURASHIGE, 1974). A concentration of 5% (w/v) has generally proved to be optimal in the culture of heterotrophic *Citrus* organs and tissues (RANGASWAMY, 1961; MURASHIGE and TUCKER, 1969; KOCHBA and BUTTON, 1974). Sucrose appears necessary for the development of immature (small) embryos (RANGASWAMY, 1961) but possibly not for mature (large) ones (OHTA and FURUSATO, 1957). Omission of sucrose prior to transferring plantlets to soil is probably advantageous as it should encourage autotrophy and thereby improve the rate of subsequent survival.

3.1.3 Amino Acids

Glycine was found to increase slightly the yield of lemon albedo callus (MURASHIGE and TUCKER, 1969) and is generally included at concentrations of 2 to 4 mg/l. Other L-amino acids and casein hydrolysate failed to improve albedo callus yields (MURASHIGE and TUCKER, 1969). Supplementation with complex substances rich in amino acids e.g. casein hydrolysate, and extracts of malt and yeast

have proved beneficial in other cases (see Sect. 3.1.5). These responses may have been due either to the supply of particular amino acids or to the provision of reduced nitrogen not included in some basal media. In view of these responses, and the changes in amino acid composition associated with bud formation (CHATURVEDI *et al.*, 1974a), further investigations into the amino acid requirements of organogenic systems is desirable.

3.1.4 Vitamins

MURASHIGE and TUCKER (1969) systematically established optimum levels of some vitamins for the proliferation of albedo callus. These levels, somewhat higher than those generally required by other genera, were as follows (mg/l): myo-inositol, 100; pyridoxin HCl, 10; nicotinic acid, 5; thiamin HCl, 10. Of these, only thiamin appeared to be essential while the following were inactive: ascorbic acid, biotin, calcium pantothenate, choline, folic acid, and para-amino benzoic acid. Ascorbic acid was found to enhance the growth of *Citrus natsudaidai* plantlets (OHTA and FURUSATO, 1957) and the stimulation of pseudobulbil production in nucellar tissue of *Citrus sinensis* (BUTTON and BORNMAN, 1971a).

3.1.5 Complex Organic Substances

Despite their incompletely-defined composition, a number of organic complexes have contributed greatly to the success of *Citrus* tissue cultures. Malt extract (ME) has been found generally to be either essential (BITTERS *et al.*, 1970) or at least beneficial to embryogenesis in nucellar cultures (RANGAN *et al.*, 1968, 1969; BUTTON and BORNMAN, 1971a; KOCHBA *et al.*, 1972; MITRA and CHATURVEDI, 1972; KOCHBA and SPIEGEL-ROY, 1973). In some systems ME can be replaced by combinations of orange juice, NAA, and adenine sulphate (RANGAN *et al.*, 1968, 1969) or by ADS and kinetin (MITRA and CHATURVEDI, 1972) and to some extent by adenine alone (KOCHBA and SPIEGEL-ROY, 1973). BUTTON and BOTHA (1975) found that ME stimulated the development of pseudobulbils from single cells derived from embryogenic "Shamouti" callus. Ovular callus proliferation was enhanced by 500 mg/l ME (RANGASWAMY, 1959) but similar concentrations were detrimental to the development of embryos within ovules, and to the growth of plantlets in culture (RANGASWAMY, 1961). In stem-callus systems, GRINBLAT (1972) found that ME suppressed callus proliferation, and was not beneficial to either bud formation or shoot growth. In a similar system derived from another *Citrus* species, CHATURVEDI and MITRA (1974) observed enhanced shoot vigour in the presence of ME + BA + NAA.

Yeast extract (YE) improved growth and organ development in cultured nucelli (SINGH, 1963) but suppressed plantlet growth (RANGASWAMY, 1961). Coconut milk (CM) has been used to great advantage in the culture of many organs, tissues, and cells (STEWARD *et al.*, 1969). In *Citrus* cultures, CM stimulated embryogenesis in cell cultures (BUTTON and BOTHA, 1975), and to a limited extent in nucellar cultures (BUTTON and BORNMAN, 1971a; KOCHBA *et al.*, 1972; MITRA and CHATURVEDI, 1972). The normal development of globular embryos was promoted by 10% CM (SABHARWAL, 1963), while high concentrations (40%)

appear harmful (RANGASWAMY, 1961). Proliferation of callus derived from flavedo tissue was stimulated greatly (BRUNET and IBRAHIM, 1973) while that derived from other fruit parts is slightly promoted by CM (STEVENSON, 1956; MURASHIGE and TUCKER, 1969). From these observations it is evident that the responses of tissues to CM varies markedly. It also appears that morphogenetic effects of CM are limited to the early phases of embryogenesis and subsequent development of globular embryos.

Casein hydrolysate (CH) alone (RANGASWAMY, 1958a, 1961), or combined with YE (SINGH, 1963) promoted the maturation of immature embryos and their further development into plantlets. In some cases however, CH appears to promote callus initiation rather than embryo development (SABHARWAL, 1963). Little stimulation of growth was detected in albedo callus cultures (MURASHIGE and TUCKER, 1969).

Orange juice appears to stimulate *Citrus* callus growth in some cases (MURASHIGE and TUCKER, 1969; THORPE *et al.*, 1971; ERNER *et al.*, 1975) and not in others (BRUNET and IBRAHIM, 1973). It also stimulated embryogenesis in cultured nucelli when added together with α-NAA and ADS (RANGAN *et al.*, 1968, 1969). The major active fraction, at least in respect of callus growth, appears to be citric acid (ERNER *et al.*, 1975).

3.1.6 Growth Regulators

In general, it appears that auxins are not essential for *Citrus* callus proliferation, particularly in explants from immature fruit tissues. Although not essential, auxins do stimulate callus growth (SCHROEDER and SPECTOR, 1957; MURASHIGE and TUCKER, 1969) and root development (OHTA and FURUSATO, 1957; KOCHBA *et al.*, 1972) but not embryogenesis (KOCHBA and SPIEGEL-ROY, 1973). In some cases 2,4-D is more active than α-NAA, which is in turn more active than IAA (MURASHIGE and TUCKER, 1969). When auxin is used together with a cytokinin, the two regulators can stimulate embryogenesis (KOCHBA *et al.*, 1972; MITRA and CHATURVEDI, 1972). Where embryogenesis is required, especially in nucellar cultures, auxin should be avoided if possible since its effects are inconsistent and dependent upon the level of other growth substances.

Cytokinins, apparently not essential (MURASHIGE and TUCKER, 1969; KOCHBA *et al.*, 1972; GRINBLAT, 1972), do enhance the proliferation of pseudobulbils (RANGASWAMY, 1958a), the stimulation of adventive bud formation (GRINBLAT, 1972; CHATURVEDI and MITRA, 1974), and the suppression of root development (GRINBLAT, 1972). Although not regarded as a true cytokinin, adenine does appear to stimulate growth and organization in nucellar cultures (RANGASWAMY, 1961; BUTTON and BORNMAN, 1971a; MITRA and CHATURVEDI, 1972; KOCHBA and SPIEGEL-ROY, 1973).

Gibberellic acid (GA_3), though not essential, does stimulate the growth of albedo callus cultures (SCHROEDER and SPECTOR, 1957; MURASHIGE and TUCKER, 1969) as well as callus derived from vegetative buds (ALTMAN and GOREN, 1974b). Embryogenesis does not appear to be stimulated by GA_3 (MITRA and CHATURVEDI, 1972), but germination and growth of large embryos is promoted (RANGASWAMY, 1961). One of the most notable effects of GA_3 in *Citrus* tissue cultures has

been the general promotion of root formation, and in some cases, shoot growth (MAHESHWARI and RANGASWAMY, 1958; RANGASWAMY, 1961; BUTTON and BORNMAN, 1971a, b; KOCHBA *et al.*, 1974).

3.1.7 Culture Conditions

Relatively little systematic research has been published on this topic, but a number of generalizations can safely be made.

Light, particularly high intensity, reduces the proliferation of callus from albedo (SCHROEDER, 1958, 1960; MURASHIGE and TUCKER, 1969; THORPE *et al.*, 1971), flavedo (BRUNET and IBRAHIM, 1973), and juice vesicles (MURASHIGE and TUCKER, 1969). Thus, unorganized callus should be maintained in darkness for optimum growth.

Cultures in which organogenesis is desired, should as a general rule, be maintained under light of ca 3000 lux for 12 to 16 h per day.

The effects of temperature on growth and organization have not been adequately studied. In the absence of more specific data, temperatures of 25° to 30° C appear optimal and are generally used.

Most *Citrus* tissue cultures have been maintained successfully on media gelled with 0.8 to 1.0% agar. There appears to be little advantage in using liquid media for the culture of organs or tissues, although STEVENSON (1956) observed more natural embryo development in such a system. Single cells proliferated better when spread on the surface of a gelled medium than when mixed in with it (BUTTON and BOTHA, 1975). VARDI *et al.* (1975) observed successful regeneration from protoplasts which were mixed in with the gelled culture medium.

3.2 Induction and Development of Citrus Embryos into Plants

It is often necessary to distinguish between zygotic embryos and those arising adventitiously (see Sects. 4. and 5.). This is usually difficult as the two classes of embryos are morphologically similar. The successful development of excised embryos into plantlets in culture (MAHESHWARI and RANGASWAMY, 1958) was achieved some 10 years before adventive embryogenesis itself was induced (RANGAN *et al.*, 1968, 1969). For the purpose of this review, the ontogenetic rather than the historical course will be followed.

3.2.1 Direct Induction of Adventive Embryos in Citrus Tissues

In view of the natural tendency of the nucellus to form adventive embryos, most instances of artificially-induced embryogenesis have been in this tissue. RANGAN *et al.* (1968, 1969) were the first to induce adventive embryogenesis directly in nucellar tissue excised from developing seeds (100 to 120 days post pollination). Using similar explants, BITTERS *et al.* (1970) found that the period during which nucelli could be excised and successfully grown could be extended, but depended on the cultivar. Both RANGAN *et al.* (1969) and BITTERS *et al.* (1970) made use of monoembryonic *Citrus* cultivars.

Embryogenesis has also been induced in nucellar tissue excised from abortive (BITTERS *et al.*, 1970) and unfertilized (BUTTON and BORNMAN, 1971a) ovules of the seedless navel orange. In the Robertson navel orange, embryogenesis occurred in excised nucelli from the time of pollination until six weeks later, at which stage embryogenesis was optimal (BITTERS *et al.*, 1970). The use of entire, unfertilized ovules of the Washington navel (BUTTON and BORNMAN, 1971a), as well as from the "Lane" and "Navel Late" clones (unpublished), extended the period during which nucellar embryogenesis occurred. Successful cultures were established using entire unfertilized ovules excised from fruits up to 40 mm diameter (3 to 4 months post anthesis). Embryogenesis has also been reported to occur in the nucellus of cultured, unfertilized ovules of polyembryonic, seed-bearing cultivars (KOCHBA *et al.*, 1972; MITRA and CHATURVEDI, 1972).

All the tissues mentioned above were cultured on modified MS media, mainly that of MURASHIGE and TUCKER (1969). The MS basal medium appears to provide significant requirements for embryogenesis. Earlier attempts at inducing embryogenesis ab initio in nucellar tissue failed on other media despite the addition of supplements which promote embryogenesis when added to the MS medium (RANGASWAMY, 1961).

Malt extract invariably promoted adventive embryogenesis in nucelli excised from fertilized (RANGAN *et al.*, 1968, 1969; BITTERS *et al.*, 1970) and unfertilized ovules (BUTTON and BORNMAN, 1971a; KOCHBA *et al.*, 1972; MITRA and CHATURVEDI, 1972). The active fraction(s) of ME are as yet unknown, but are likely to include one or more amino acids and possibly a cytokinin (VAN STADEN, 1974). There are a number of other substances which have promoted embryogenesis in nucellar cultures. These include adenine (BUTTON and BORNMAN, 1971a), combinations of adenine sulphate and kinetin (MITRA and CHATURVEDI, 1972), and a combination of orange juice, α-NAA, and ADS (RANGAN *et al.*, 1968, 1969).

It is significant that adventive embryos will not develop in excised nucelli taken from fertilized ovules of all monoembryonic cultivars (BITTERS *et al.*, 1970; BUTTON, unpublished). Entire, unfertilized ovules of a Shaddock and the Ellendale mandarin have also failed to yield adventive embryos (BUTTON and KOCHBA, unpublished). A potent antiembryogenic substance from nucellar cells of monoembryonic *Citrus* cultivars has recently been discovered by Esan (see MURASHIGE, 1974). Detailed information has not yet been published but the presence of this substance is undoubtedly also a major factor limiting the initiation of nucellar embryos in vitro.

3.2.2 Indirect Induction of Adventive Embryos

It appears that most cultured nucellar embryos, whether induced ab initio or not, give rise to further embryos. Many of these additional embryos arise from the hypocotyl zone of existing ones. Others develop from pseudobulbils (white or pale green spherical bodies) or from varying amounts of callus associated with embryos formed initially (MAHESHWARI and RANGASWAMY, 1958; RANGASWAMY, 1958a, 1961; SABHARWAL, 1963; KOCHBA *et al.*, 1972).

MITRA and CHATURVEDI (1972) observed embryo initiation in callus which proliferated from the walls of unpollinated ovaries of two polyembryonic species. Like nucellar embryos, these too gave rise to others from the hypocotyl zones.

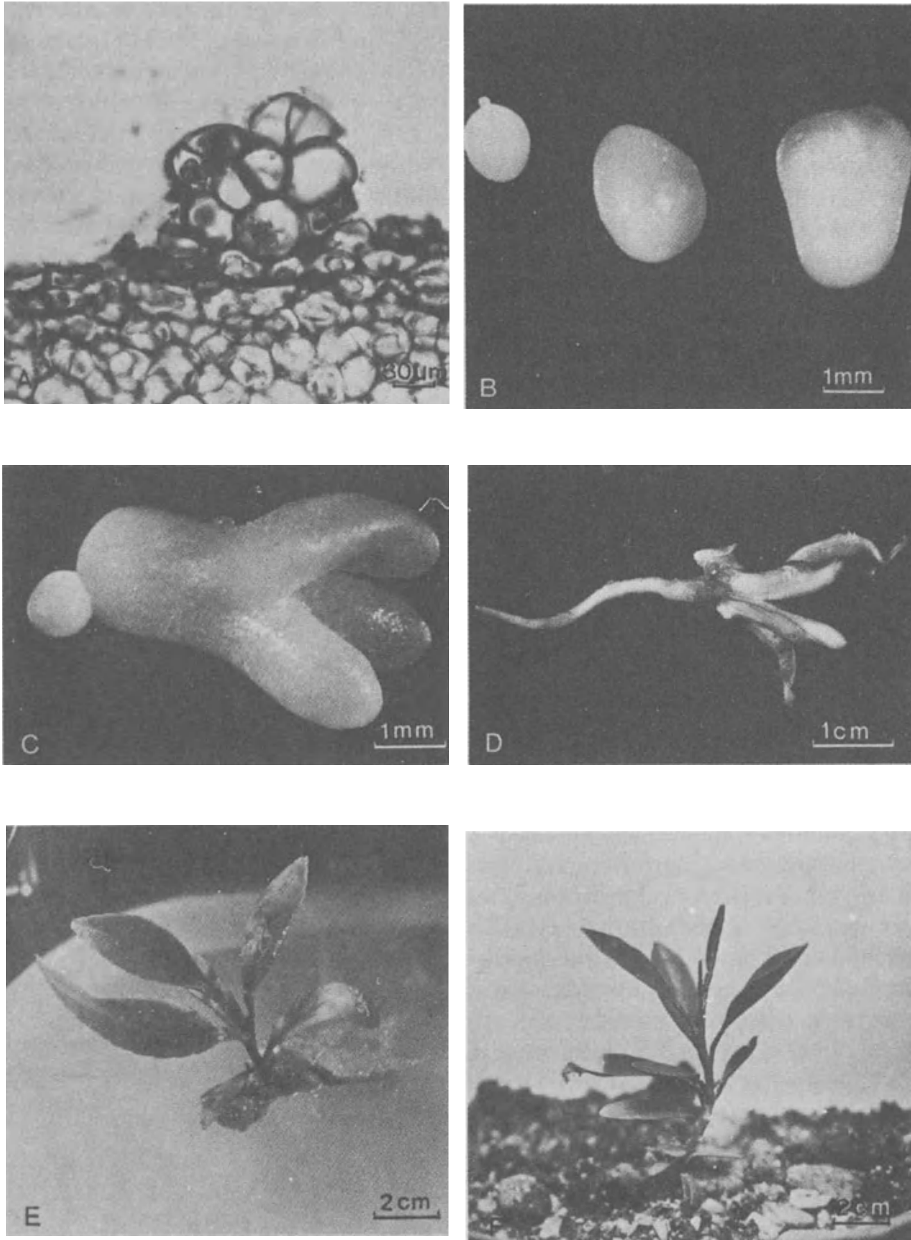

Fig. 1A–F. Various stages in the development of "Shamouti" plantlets from embryogenic callus. (A) Small proembryo developing on the surface of a pseudobulbil. (B) and (C) Stages in embryo development. Note the multiple cotyledons and additional pseudobulbil attached to the larger embryo (C). (D) Rooted embryo with abnormal cotyledons. (E) Normal plantlet in a flask prior to transfer to soil. (F) Young plant growing in soil

KOCHBA *et al.* (1972) obtained an embryogenic callus from entire, unfertilized ovules and isolated nucelli of "Shamouti" orange *(C. sinensis)*. A similar callus was derived from sliced, unfertilized ovules of *Citrus aurantifolia* (MITRA and CHATURVEDI, 1972). MITRA and CHATURVEDI noted a decrease in embryo formation after repeatedly subculturing their callus on media supplemented with ADS, kinetin, and NAA. In the "Shamouti" callus however, KOCHBA and SPIEGEL-ROY (1973) observed continued embryogenesis which was promoted by ME and AD.

After repeated subculturing a number of "Shamouti" callus lines differing greatly in their embryogenic potential were selected. These lines all became independent of exogenous auxin and cytokinin addition (KOCHBA and SPIEGEL-ROY, unpublished). Embryo formation in callus lines of low embryogenic potential was strongly stimulated by ageing the callus prior to subculture. A similar response was observed when callus was maintained on a medium devoid of sucrose prior to subculture (KOCHBA and BUTTON, 1974). Stimulation of embryo differentiation was also observed when the callus was subjected to fairly high doses of gamma irradiation (SPIEGEL-ROY and KOCHBA, 1973a). Maximum stimulation was obtained by 16 K rad and only doses of 28–32 K rad were lethal. Irradiation of medium alone, when containing malt extract caused as much embryo stimulation as did irradiation of the callus alone. In media containing adenine, irradiation inhibited callus growth and embryo formation while on media containing low levels of kinetin and IAA only, callus growth was depressed.

Light and electron microscope studies on "Shamouti" callus (BUTTON *et al.*, 1974) showed that this tissue comprises small globular proembryos or meristemoids (TORREY, 1966) at various stages of development and is devoid of unorganized parenchymatous tissue. Embryogenesis occurs in single cells on the periphery and within existing proembryos. Proembryos may enlarge into spherical pseudobulbils up to 4 mm in diameter which rarely develop into plants. Alternatively they can develop along classical lines into mature embryos and later into plantlets. Embryos are in many cases multicotyledonous with varying degrees of fasciation.

In the light of these findings one can regard conditions stimulating embryo formation as those causing a shift from continuous proembryonic proliferation (callus-like in appearance) towards embryo formation and subsequent plant development. Factors causing this shift are now under study.

Since plantlets are derived from single cells this culture system holds great promise for the production of solid mutants and their screening. More recent work has shown that enzymatically separated single cells (BUTTON and BOTHA, 1975) and protoplasts (VARDI *et al.*, 1975) derived from this callus retain the embryogenic potential in colonies arising from them. This further demonstrates the great extent to which virtually all cells of this callus are able to express their totipotency.

Totipotency, the autonomous growth pattern, eliminating complications created by the need to add one or more growth regulating substance to the culture medium, and the selection of lines of different embryogenic potential turn the "Shamouti" callus into an interesting model system for the study of factors controlling proembryo, embryo, and plant development.

3.2.3 Development of Zygotic and Adventive Embryos into Plants

As can be expected, the greater the extent of embryo maturity, the less critical is its requirement for specific substances and conditions. The initial stages of embryogenesis demand the most precise conditions which have been determined in some cases. Immature zygotic embryos appear to be less amenable to culture than adventive ones at the same morphological stage of development. Early heart-shaped but not globular embryos have been successfully cultured in vitro (RANGAN *et al.*, 1968, 1969).

The successful culture of mature *Citrus* embryos (probably nucellar) was first achieved by OHTA and FURUSATO (1957) on White's medium. Within a few years, numerous reports appeared on the development in vitro of globular embryos embedded in, and excised from nucellar tissue (MAHESHWARI and RANGASWAMY, 1958; RANGASWAMY, 1958a, b, 1959, 1961; SABHARWAL, 1963; SINGH, 1963). In some instances, the ultimate development of entire plants was reported. Addition of CH to media has frequently been reported to enhance the generation of further pseudobulbils and embryos from existing ones (RANGASWAMY, 1958a, 1961; SABHARWAL, 1963). It also appears to promote the development of individual embryos (RANGASWAMY, 1961; SABHARWAL, 1963; SINGH, 1963, RANGAN *et al.*, 1968, 1969).

Fully developed embryos germinate and grow normally in media lacking carbohydrate. In some cases (OHTA and FURUSATO, 1957) the inclusion of sucrose in the medium actually depressed seedling growth.

A small proportion of fully developed embryos form roots and shoots on basal media with supplements such as ME and YE (RANGASWAMY, 1961; SABHARWAL, 1963; SINGH, 1963). The incorporation of GA_3 into the medium however, greatly enhances the development of roots in fully developed (RANGASWAMY, 1961; BUTTON and BORNMAN, 1971a; KOCHBA *et al.*, 1972) and partially developed embryos (KOCHBA *et al.*, 1974). The addition of ADS (KOCHBA *et al.*, 1974) also enhances root development.

Shoot development normally occurs spontaneously after root development has commenced but we have observed that in many cases roots develop but shoots do not (BUTTON *et al.*, 1974). This remains a problem which is receiving our attention. Once shoot development has commenced, plantlets should be transferred to another medium lacking GA_3 to avoid the development of weak, spindly plants (BUTTON and BORNMAN, 1971b).

Small plantlets are generally grown on gelled media comprising only the inorganic nutrient elements. Higher than normal concentrations of iron (chelated) are beneficial in preventing chlorosis. Plantlets grown in culture are poorly adapted to the external environment and many succumb shortly after being transferred to soil (SINGH, 1963; BITTERS *et al.*, 1970; BUTTON and BORNMAN, 1971a).

Losses can be minimized by transplanting plantlets into sterilized potting mixture which is saturated with the BM mineral nutrient solution. Plantlets should then be covered with a bell jar (CHATURVEDI and MITRA, 1974) or enclosed in transparent polythene bags (NAVARRO *et al.*, 1975) until they have developed a suitable root system. Enclosing structures are then removed and the plantlets further hardened according to standard procedures.

Citrus plants can thus be obtained with great success from suitable nucelli or immature embryos by utilizing the following sequence: (1) Nucellus/unfertilized ovule inoculated onto the basal medium (BM) of Murashige and Tucker (1969) + 500 mg/l ME. (2) Embryos separated and placed on BM + 1 mg/l GA_3. (3) Rooted embryos transferred to BM containing double Fe concentration. (4) Plantlets transplanted into sterilized potting mixture moistened with inorganic solution of BM, and enclosed in transparent polythene bags. Plants placed in shade. (5) Polythene bags removed in stages, soil kept moist with tap water. (6) Plants gradually moved into full sunlight.

Citrus plantlets derived in tissue culture tend to grow poorly on their own roots, even after they have been successfully established in soil. Attempts are currently being made to improve the rate of growth by grafting shoots from plants in tissue culture onto normal seedling rootstocks. Results have been encouraging provided care is taken in conditioning the tender scion immediately after grafting [steps 4 (modified), 5, and 6].

3.3 Initiation and Culture of Vegetative Buds

Adventitious buds were induced in callus cultures derived from stem tissue of seedlings grown in vitro (GRINBLAT, 1972; CHATURVEDI and MITRA, 1974). In both instances buds developed into normal shoots which were then transferred to a second medium for the induction of roots. As expected, benzyl adenine promoted bud formation but depressed or prevented rooting. Roots were induced on a medium containing auxin. Marked qualitative and quantitative changes in amino acid composition of the cultures was correlated with bud formation (CHATURVEDI *et al.*, 1974a, b). Unfortunately the lack of sufficient anatomical and cytological detail prevented them from determining whether these changes were the cause or result of bud initiation.

Excised vegetative buds have been grown aseptically to study the effects of applied growth regulators (ALTMAN and GOREN, 1971, 1974a, b). It was found (1974b) that sprouting of buds in vitro was affected by regulators in a similar way to buds in vivo. Sprouting was retarded by IAA and ABA in the medium, but slightly enhanced by GA_3. The sprouting of numerous shoots from single explants was induced by BA and kinetin, while GA_3 caused the new growth to have excessively long internodes. The similarity in response between buds grown in vivo and in vitro is significant. It permits the study in vitro of factors controlling complex phenomena such as flower bud induction and development in vivo.

3.4 Physiological Disorders of Fruits

There are a number of physiological disorders in *Citrus* fruits which result in a high proportion of decay and consequent financial loss. An in vitro system is particularly attractive in the study of such disorders because of the complexity of the interacting factors. In addition, in vitro cultures permit the year-round study of normally seasonal problems (ALBRIGO, 1970). The fact that most fruit tissues

can be easily cultured is a further attraction. Rind breakdown (EAKS, 1969) creasing (CHAPMAN, 1968), granulation (ERICKSON, 1968) and drying out (VAN NOORT, 1969), are all disorders about which relatively little is known because of the complex factors causing them. Creasing for example, is probably due to excessive continued endocarp expansion after cell division in the mesocarp (albedo) has ceased. This causes rupturing of the albedo which makes the fruit particularly susceptible to fungal or bacterial infections. GILFILLAN *et al.* (1974) have shown that GA_3 sprays can greatly reduce the incidence of creasing. This reduction may well be due to the promotion of cell division in the albedo by GA_3 as described in an in vitro system by SCHROEDER and SPECTOR (1957). An in vitro study would undoubtedly help in the understanding and solving of this problem. Numerous other callus cultures derived from fruit tissues have been established and used to study basic morphological, cytological, and physiological phenomena (SCHROEDER and SPECTOR, 1957; SCHROEDER, 1958, 1960, 1961; ALLAN, 1962; KORDAN, 1963a, b, c, 1965a, b). However, very few of these have so far been put to practical use.

3.5 Nutritional Disorders of Citrus Trees

Deficiency and toxicity symptoms in leaves and fruits reflect advanced stages of nutrient imbalance when yields and fruit quality are already affected. The advent of leaf analysis has done much to forestall the development of undesirable nutrient conditions. It has also helped in rectifying nutritional problems in the most efficient and inexpensive ways (STANTON, 1966). In some cases the physiological and anatomical effects of imbalances are known. In others however, only the end result is seen because of complex interactions nearer to the source of the problem. Tissue culture could prove a valuable tool in studying the underlying physiological causes of and morphological responses to, nutrient imbalances. This applies particularly to systems where easily cultured fruit tissues can be used.

BAR-AKIVA *et al.* (1974) for example, created an in vitro model in an attempt to solve the problem of thick, coarse peels caused by high levels of potassium. They found in *Citrus*, as in other higher plants, that low K^+ was associated with high concentrations of putrescine and vice versa. By adding putrescine to a nutrient medium high in K^+, they were able to reduce greatly the proliferation of peel callus. When putrescine was applied to trees in orchards where K^+-induced thick peels was a problem, they observed a marked reduction in mean rind thickness and in the incidence of fruit with thick rinds. It remains to be seen whether putrescine applications are an economic solution to the problem or whether trees can be encouraged to produce their own putrescine by other means.

3.6 Interrelationship with Pathogens

In recent years, basic studies of the effects of *Citrus* viruses on the cytology (KORDAN, 1963a) and biochemistry (FELDMAN, 1969; GRASSO *et al.*, 1972; HANKS and FELDMAN, 1972) of the host tissues have been made. KORDAN (1963a) made a detailed cytological comparison between calli derived from juice vesicles taken

from fruits borne on Exocortis-infected, and Exocortis-free trees. He was unable to detect cytological differences between these calli, probably because this virus affects *Poncirus* and its hybrids, but is tolerated by *Citrus* trees. Symptoms of Exocortis are not seen in fruits, while those of Stubborn (CHAPOT, 1961) and Greening diseases are clearly expressed. A cytological study of calli derived from fruits of trees infected with Stubborn or Greening would probably reveal interesting results.

Tissue, cell, and particularly protoplast cultures (ZAITLIN and BEACHY, 1974), afford excellent systems for detailed studies on relationships between host and virus, mycoplasma, and other pathogenic diseases. Significant advances in this field can be expected in the near future, since single cells (BUTTON and BOTHA, 1975) and protoplasts (VARDI *et al.*, 1975) of *Citrus* can be obtained, cultured, and reorganized into plants. Particularly significant is the possibility that any biochemical, cytological, or other differences found during detailed in vitro studies could be used as rapid indexing techniques. These techniques could be extended further to screen for resistant strains or at least to determine factors associated with resistance.

3.7 Synthesis of Secondary Metabolites

The commercial production of secondary products from *Citrus* tissue cultures is not a practical proposition. All commercially valuable secondary products such as asorbic acid, essential oils, flavones, hespiridin, and pectin, occur in the nonjuice fraction of the fruits. They are effectively and economically extracted as by-products from juicing factories. Furthermore, the concentrations of at least some of these substances is lower in cultured tissues than in fresh tissues (BRUNET and IBRAHIM, 1973). Tissue cultures of the flavedo could prove useful in the study of essential oil biosynthesis (SCHROEDER, 1958; BRUNET and IBRAHIM, 1973).

4. The Elimination of Citrus Virus Diseases and Clone Rejuvenation

Clonal senescence is probably the result of a combination of natural physiological ageing, para-mutation, and virus infections not showing acute symptoms (CAMERON and FROST, 1968). The exact extent to which virus diseases contribute to the decline in vigor and productivity of clones is not known. However, much of the industry is now dependent upon selected nucellar bud-lines of old clones. These bud-lines are physiologically rejuvenated and are largely, if not entirely free from virus diseases. They result in trees that are commonly more vigorous, longer-lived, and higher yielding than their parent lines. They also make possible the establishment of desirable rootstock-scion combinations which would fail if infected, old clone scions were used (CAMERON and FROST, 1968). To date there are at least 15 known *Citrus* viruses (ANONYMOUS, 1968) but many more probably exist which do not result in the appearence of acute symptoms.

4.1 Distribution and Economic Significance of Virus Diseases

Citrus virus diseases occur to varying degrees throughout the citrus-producing areas of the world. These virus diseases are primarily transmitted by budding, the standard method of propagation. A number of the viruses are also spread by effective insect vectors. Long distance dissemination has been due mainly, if not exclusively, to the transfer of infected budwood. Effective indexing procedures (ANONYMOUS, 1968) have greatly helped in controlling further dissemination of known viruses (REUTHER *et al.*, 1972).

Virus diseases are responsible for great reductions in yield, tree vigor, and longevity. Some of the losses caused by viruses have been catastrophic. For example, in the Brazilian state of Sao Paulo, tristeza destroyed more than six million trees in twelve years (COCHRAN, 1959). A further 10 million trees were destroyed by the same virus in one province of Argentina (VALIELA, 1961). They have also confounded results obtained from many rootstock-scion experiments (REUTHER *et al.*, 1972).

In view of these problems, expensive cultivar-improvement programs exist in all major producing countries. In the state of California alone, between the years 1961 and 1972, some $1·5 million was spent on variety improvement (REUTHER *et al.*, 1972). These programs are very largely concerned with the elimination of viruses from proven clones, and the establishment of virus-free bud-lines. Some viruses can be eliminated by careful heat treatment of budwood (BITTERS and MURASHIGE, 1967) but most have to be eliminated by other means such as the selection of nucellar lines (KNORR and CHILDS, 1968).

4.2 Importance of Nucellar Lines

It is generally accepted that virus diseases of *Citrus* are very rarely seed-transmitted (BITTERS and MURASHIGE, 1967). CHILDS and JOHNSON (1966) have detected seed-transmission of psorosis in Carizzo Citrange, a *Poncirus* × *Citrus* hybrid, but no report on seed-transmission within the genus *Citrus* has been forthcoming. Virus particles are generally restricted to the host vascular tissue, particularly the phloem. Since there is no direct vascular connection between the parent and either the zygotic or nucellar embryos, virus particles are eliminated from seedling offspring.

4.2.1 Nucellar Lines from Seeded Polyembryonic Clones

Most *Citrus* cultivars fall into this category. Virus elimination from these clones can be readily accomplished by controlled pollination with marker pollen and the subsequent selection of true-to-type seedlings (KNORR and CHILDS, 1968). Pollen from *Poncirus trifoliata* is used, as it imparts a dominant trifoliate leaf character to all hybrid seedlings which can then be eliminated.

4.2.2 Nucellar Lines from Seeded, Monoembryonic Clones

A number of commercially important *Citrus* clones are virtually or completely monoembryonic, and thus cannot be freed from viruses and rejuvenated naturally

via nucellar seedlings. The potential of using tissue culture in such cases as a means of artificially inducing and culturing nucellar embryos in vitro has long been appreciated (RANGASWAMY, 1958a, b, 1961; MAHESHWARI and RANGASWAMY, 1958; BITTERS *et al.*, 1970), but has only recently been achieved.

RANGAN *et al.* (1968, 1969), successfully obtained nucellar plants from three monoembryonic cultivars by eliminating zygotic embryos and culturing the surrounding nucellus of developing seeds, 100 to 120 days post pollination. Adventive embryos developed in a maximum of 20% of the nucellar explants on a basal medium with malt extract. Embryos developed into plantlets which were subsequently transferred to soil. Unfortuanately, this technique has not proved successful with all monoembryonic clones (BITTERS *et al.*, 1970; BUTTON, unpublished). In addition, the period during which nucellar explants are amenable to culture is very restricted (RANGAN *et al.*, 1968, 1969; BITTERS *et al.*, 1970). Entire unfertilized ovules (and their nucelli) of monoembryonic clones are potentially useful as starting material but have so far proved unamenable to culture (MITRA and CHATURVEDI, 1972; BUTTON and KOCHBA, unpublished).

4.2.3 Nucellar Lines from Seedless Clones

Seedlessness in *Citrus* is a commercially desirable trait, but one which precludes the natural production of nucellar seedlings. The degree of seedlessness is variable and in many potentially polyembryonic clones occasional seeds for the purpose of establishing nucellar lines can be obtained by controlled pollination. Other cultivars such as the navel orange however, are virtually completely seedless as a result of complete pollen sterility and frequent megaspore abortion (FROST and SOOST, 1968).

BITTERS *et al.* (1970, 1972) derived virus-free seedlings of Robertson navel orange from nucellar isolates in vitro. Cultures were initiated from immediately after pollination until six weeks later when best results were obtained. Beyond this period, complete ovule degeneration prevented the establishment of further cultures.

BUTTON and BORNMAN (1971a) succeeded at inducing embryogenesis in nucellar explants and in nucelli within unfertilized ovules of Washington navel orange. Entire ovules were not only most easily obtained and handled, but yielded more embryos per culture than did nucellar isolates. Furthermore, embryogenesis in three navel orange clones occurred readily in ovules removed from fruits of up to 40 mm diamter (3 to 4 months post anthesis). Nucellar explants from such large fruits invariably failed to proliferate. Thus the use of entire, unfertilized ovules extends the period during which nucellar cultures can be successfully initiated.

More recently (BUTTON, unpublished), this technique has been used to import two navel orange clones into South Africa with very little danger of inadvertent virus importation. These clones have been vegetatively propagated on a number of rootstocks and are now in trial orchards.

The development of plantlets from unfertilized ovules of potentially seeded, polyembryonic cultivars have also been reported (KOCHBA *et al.*, 1972; MITRA and CHATURVEDI, 1972). In such cultivars however, virus-free nucellar lines can

be obtained more easily through controlled pollination and subsequent seedling selection.

All attempts at deriving plantlets from unfertilized ovaries (MITRA and CHATURVEDI, 1972), unfertilized ovules and nucelli excised from such ovules (BUTTON and KOCHBA, unpublished) of monoembryonic cultivars have failed.

4.3 Adventive Embryos from Other Tissues

Most tissue cultures aimed at obtaining virus-free lines have involved the nucellus because of its natural, frequent tendency to produce adventive embryos. However, other tissues have also been used to this end. The ovary wall appears to be the only one in which embryogenesis has been induced directly.

MITRA and CHATURVEDI (1972) observed rapid development of embryos from the walls of unpollinated ovaries of *C. aurantifolia* and *C. sinensis*, both polyembryonic species. Embryos which were morphologically similar to those derived from nucellar cultures, were successfully grown into plants. It is likely, although unproven, that these plants will also exhibit juvenile characteristics such as thorniness and delayed bearing.

4.4 Plantlets from Nonnucellar Callus Cultures

Callus cultures have been developed from many fruit tissues, leaves, buds, and seedling stems. However, only those from leaves and young seedling stems have organized into plants. GRINBLAT (1972) using *Citrus madurensis*, and CHATURVEDI and MITRA (1974) using *Citrus grandis* and *Citrus sinensis*, obtained callus from stem explants taken from young seedlings grown in vitro. GRINBLAT regenerated plants from callus attached to the original explants while CHATURVEDI and MITRA developed plantlets from subcultured callus. These systems are of dubious practical value in the production of virus-free lines since explants were derived from seedlings (already virus-free), and not from proven clones. It remains to be seen whether callus can be obtained and organized from stem tissue of clonally propagated mature trees.

4.5 Stem Tip Cultures

So far all attempts to develop *Citrus* plantlets from bud meristems and small apices in culture have failed. However, entire buds (ALTMAN and GOREN, 1971, 1974a, b) and bud apices (MURASHIGE *et al.*, 1972) can be maintained in culture for several months.

MURASHIGE *et al.* (1972) have developed a technique of aseptically grafting shoot apices (meristem dome plus four to six leaf primordia) onto young seedling rootstocks in vitro. The proportion of successful grafts ranged from 5 to 40% and resulted in the development of a few virus-free plants. NAVARRO *et al.* (1975) grafted smaller shoot tips (meristem dome plus three leaf primordia) onto etiolated Troyer citrange seedlings grown in vitro. Scion tips were taken from actively growing shoots of old-clone trees. A number of important *Citrus* clones have been

freed from Tristeza, Psorosis, Exocortis, and Stubborn disease in this way. The great advantage of this process over nucellar cultures is that virus and virus-like diseases can be eliminated without the reappearance of juvenile features.

5. Uses of Tissue Culture for Citrus Breeding

Breeding of perennial species such as fruit trees is beset with many problems. Commercial fruit trees, especially outbreeding species are highly heterozygous and have remained unchanged for generations because they have been vegetatively (clonally) propagated. Vegetatively propagated fruit trees are also characterized by a more or less prolonged juvenile period during which they are either unfruitful or bear fruits uncharacteristic of the mature tree. Juvenility becomes even more pronounced when trees are propagated from seeds—a necessary procedure when hybridization is the adopted method of breeding. Mutation breeding, as an alternative approach, reduces juvenility when mature tissues are used but creates other problems such as the formation of chimaeras.

5.1 Breeding by Hybridization

5.1.1 Special Problems Involved in Citrus Hybridization

In *Citrus*, controlled hybridization as well as mass selection from open pollinated seedlings is further complicated and made impractical by the existence of nucellar embryos in the seeds of many cultivars. In most cases these embryos breed true to type and reduce genetic variability in seedling populations. Breeding by hybridization is, therefore, only effective when the maternal parent is monoembryonic. Unfortunately, many potential parent cultivars with desirable characters are polyembryonic and thus unsuitable for hybridization. Other cultivars have very few or no viable seeds in their fruit due to ovule or early seed abortion. Despite being a commercially desirable character, it precludes their use as maternal parents of hybrids.

5.1.2 The Role of Tissue Culture in Citrus Hybridization

Polyembryonic cultivars usually contain a zygotic embryo at the earlier stages of ovule and subsequent seed development. Nucellar embryos are most vigorous and so inhibit the full development of the zygotic embryo and cause its degeneration prior to seed maturation. By culturing the excised zygotic embryo in vitro its further development can be secured.

The culture of embryos from polyembryonic mature seeds was first attempted by OHTA and FURUSATO (1957). They assumed that the smallest embryos were the zygotic ones. However, these did not develop any further in culture. We doubt the validity of this assumption as it is well known (FROST and SOOST, 1968) that not all nucellar embryos develop simultaneously and do not reach the same stage of development in mature seeds.

The culture of correctly identified zygotic embryos was successfully achieved by RANGAN *et al.* (1969). The embryos were excised from immature seeds (100-120 days after anthesis) of the polyembryonic sour orange *Citrus aurantium*. Embryos which had reached the heart-shaped stage when excised, developed further into mature embryos, and later into plants. More immature embryos were not successfully cultured. To ensure proper identification of the zygotic embryo, flowers from which seeds were later taken were pollinated by the trifoliate orange *Poncirus trifoliata* which conveys a dominant character of trifoliate-shaped leaves useful as a genetic marker to its offspring (CAMERON and FROST, 1968).

For the successful application of zygotic embryo culture in breeding, the marker pollen would have to be substituted by that from the desired pollen parent. This creates a new problem because it was observed that the pollen parent has a strong influence on the rate of development of the zygotic embryo. *P. trifoliata* induces very vigorous development while many *Citrus* cultivars do not (FROST and SOOST, 1968). It is our observation (unpublished) that sour orange seeds obtained after *P. trifoliata* pollination, give rise to 60% zygotic seedlings as opposed to 0-10% when open pollinated. A vigorous zygotic embryo apparently inhibits or retards the development of nucellar embryos in these seeds. This is also indicated from the observation of RANGAN *et al.*, (1969) that nucellar embryos had not yet been found in the developing seeds 120 days after anthesis, while the zygotic embryo had already reached the heart-shaped stage. In seeds from open pollinated (mostly self-pollinated) sour orange fruits nucellar embryos appear earlier and are well developed after 3-4 months. In most cases the development of the zygotic embryo is retarded.

5.1.3 Somatic Hybridization of Citrus

Recent developments in the field of protoplast culture (See COCKING, 1972; BAJAJ, 1974) open new lines of thought. The fusion of protoplasts and subsequent regeneration of plants may provide another method for increasing genetic variability by producing hybrids from species which could not otherwise be crossbred. There are a few cases in which plants have been obtained from protoplasts, and only two instances of somatic hybridization are known (for details see Chap. IV. 1,2. of this Vol).

The regenerative capacity is a prerequisite for the formation of somatic hybrid plants. It is therefore of importance that protoplasts from an embryogenic nucellar callus of the Shamouti orange were successfully cultured by VARDI *et al.* (1975) and that this embryogenic capacity was retained. Somatic hybridization studies with the Shamouti protoplasts are now under way. Somatic hybridization and subsequent polyploidy should not adversely affect plant establishment in *Citrus* as polyploid cultivars are successfully grown.

5.2 Mutation Breeding in Citrus

Many of the improved *Citrus* cultivars originated as natural bud mutations. This justifies the hope that new varieties can be obtained by increasing the incidence of mutations with mutagenic agents such as ionizing radiation(s) or chemical muta-

gens. However success in mutation breeding with vegetatively propagated crops has usually been surprisingly small (NYBOM, 1961).

A mutation is an event in a single cell, which, if it is meristematic will give rise to a mutated cell-line while adjacent cells remain unchanged. The mixture of mutated and unmutated cells (a chimaera) can rarely be avoided when multicellular organs such as seeds or buds are subjected to mutagenic treatments. Depending on the proportion and size of the mutated cell line in an organ such as a shoot forming lateral buds, the phenotypic expression of the mutant character is prone to instability. Furthermore due to the loss of vigor caused by many mutations, the mutated cell lines tend to be outgrown by the normal tissue and thus never come to expression, a phenomenon termed diplontic selection (NYBOM, 1961).

Procedures of recurrent budwood selection have been designed to resolve chimaeras and to overcome loss of mutations by diplontic selection (LAPINS, 1973; VISSER, 1973). Although quite effective these procedures are cumbersome and expensive.

5.2.1 The Use of Unorganised Tissues

The loss of mutations due to chimaera formation and diplontic selection could be effectively overcome by applying the mutagenic treatment to the first cell destined to give rise to a plant. Therefore the culture of ovules and nucellar isolates from *Citrus* cultivars can be of great importance provided that embryos in these cultures are of single cell origin and that viable plants can later be established. The culture of ovules and nucelli containing partly developed embryos was carried out by MAHESHWARI and RANGASWAMY (1958), RANGASWAMY (1958a, b, 1961), SABHARWAL (1963), and SINGH (1963). Such multicellular organs are of little use in mutation breeding.

The successful culture of undifferentiated nucelli from fertilized ovules was carried out by RANGAN *et al.* (1969). Plants developed directly from the nucellar tissue and no callus was formed.

Although fertilization was previously considered a prerequisite for the successful culture of ovules and nucelli (MAHESHWARI and RANGASWAMY, 1958) this was achieved with unfertilized ovules by BITTERS *et al.* (1970), BUTTON and BORNMAN (1971a), KOCHBA *et al.* (1972) and MITRA and CHATURVEDI (1972), for different cultivars in each case. Plants developed either directly from the explant cultured (BUTTON and BORNMAN 1971a; KOCHBA *et al.*, 1972) or from a callus proliferating from the explant (KOCHBA *et al.*, 1972; MITRA and CHATURVEDI, 1972). MITRA and CHATURVEDI (1972) were also able to establish an embryogenic callus from tissues of the ovary wall.

Most of the studies described above were carried out with the aim of producing virus-free clones (RANGAN *et al.*, 1969; BUTTON and BORNMAN, 1971a; MITRA and CHATURVEDI, 1972) but these techniques can also be utilized for mutation breeding by either irradiating the unorganized nucellar explants or the unorganized callus prior to embryo formation. Irradiation of unfertilized and undifferentiated nucelli was carried out by SPIEGEL-ROY and KOCHBA (1973b) but the low

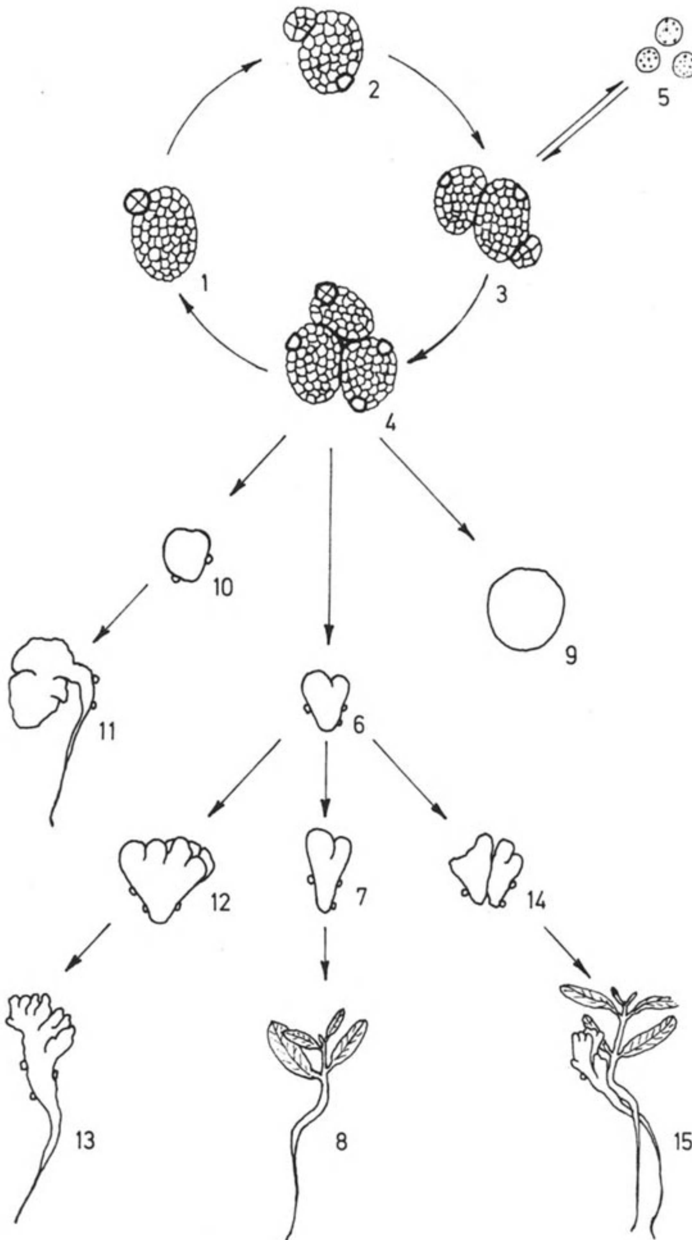

Fig. 2. Diagram of embryogenic callus proliferation (*1*, *2*, *3*, *4*, *1*), enzymic separation and regeneration from single cells and protoplasts (*5*), formation of large pseudobulbils (*9*) which occasionally develop roots (*10*, *11*). Normal plantlets (*8*) develop along classical lines from proembryos (*4*) through heart-shaped embryos (*6*, *7*). Fused plantlets (*6*, *14*, *15*) and abnormal ones with fasciated cotyledons (*6*, *12*, *13*) may also develop. Small nodules attached to the larger structures represent proembryos arising from single surface cells. Their subsequent development does not appear to be influenced by the parent structure

number of plant formation, combined with the tedious procedure of nucellus isolation, made this approach impracticable for large scale experiments. On the other hand a subcultured embryogenic callus can be used effectively and independently from seasonal restrictions provided the single-cell origin of the embryos can be established.

The single-cell origin of embryos developing in the callus of nucellar origin from the "Shamouti" orange *C. sinensis* was established by BUTTON *et al.*, (1974) and the establishment of plants from these embryos was achieved by the addition gibberellic acid and adenine sulfate to the growth medium (KOCHBA *et al.*, 1974).

The embryogenic callus of "Shamouti" orange is now used as a tissue-culture system for mutation breeding (SPIEGEL-ROY and KOCHBA, 1973b) and as a model (Fig. 2) for the study of factors controlling embryogenesis and development (see Sect. 3.2.2 for details). Gamma irradiation over the range of 1–32 K rad was applied to explants of this callus. Embryo formation increased in irradiated cultures, maximum response being observed at 16 K rad (SPIEGEL-ROY and KOCHBA, 1973a). Plants arising from the irradiated cultures are now grown for mutant selection.

5.2.2 Mutant Selection

Effective selection of mutants produced by irradiation of cultures is necessary for the complete utilization of this approach in mutation breeding. Mutations concerning fruiting habits and characters will have to be selected from bearing trees in the absence of early criteria of selection. To shorten the juvenile phase topgrafting of mature trees can, and has in fact already been successfully applied (SPIEGEL-ROY and KOCHBA, 1973b). Dwarf and compact mutants which are of great value can be selected relatively easily from young (1–2 year old) mutant populations.

The evident totipotency of cells of the "Shamouti" orange nucellar callus, even when cultured as single protoplasts (VARDI *et al.*, 1975) or as single cells (BUTTON and BOTHA, 1975) open the possibility of applying methods used in microbial mutant selection (DEVREUX, 1973; HEINZ, 1973a). Mutants which are marked by changes in their biochemical pathway or by their resistance to toxic substances in the plating medium can thus be selected (CHALEFF and CARLSON, 1974). The immediate application of this approach would be in the field of disease resistance. For example, tobacco cells resistant to the wildfire disease caused by the bacterial pathogen *Pseudomonas tabaci* have been recovered (CARLSON, 1973) by adding a toxin analogue to the medium. Direct selection for resistance to pathogens (BRAUN and LIPETZ, 1966) and virus (OTSUKI *et al.*, 1972) should be possible.

The culture of haploid totipotent cells is of great theoretical and practical importance (see Chap. II.1 of this Vol.). Recessive mutations can be recovered, and genetical analysis and therefore breeding would greatly improve by the culture of haploid plants which, by diploidization with colchicine, would provide completely homozygous plants. Attemps to obtain haploid cultures from *Citrus* spp. have failed so far (KOCHBA and SPIEGEL-ROY, unpublished). In view of the importance of the matter the culture of haploid cells in *Citrus* should be a subject to which more attention is given.

6. Conclusions

Tissue culture is an important technique in a number of facets of the citrus industry. Some of the uses have already been exploited while others are still being explored. The use of tissue cultures in studying various physiological problems has received some attention. Tissue culture provides a convenient system for systematically studying selected factors, but results may not hold good for similar systems in vivo. The study of plant/pathogen interrelations is greatly facilitated by the use of tissue or protoplast cultures. Observations on tissues or cells in culture could provide a quick means of indexing for viruses and screening for resistant genotypes.

Propagation of *Citrus* and virus elimination have been achieved by the use of tissue cultures. As a general rule, where tissue culture is used as a means of vegetative propagation, the period of unorganized growth should be kept as short as possible to minimize the chances of genetic change (MURASHIGE *et al.*, 1967). For this reason callus cultures, particularly long-term ones, should be avoided. Nucellar cultures from fertilized and unfertilized ovules have provided a means of eliminating virus diseases and rejuvenating old *Citrus* clones which do not produce nucellar seedlings naturally. They also facilitate the safe and simple transfer of such clones from one country to another. The use of entire, unfertilized ovules is advantageous in some cases as it extends the period during which nucellar plantlets can be obtained. Unfortunately, nucellar plants, whether produced in vivo or in vitro, exhibit some undesirable juvenile characteristics such as thorniness and delayed sexual maturity. For these reasons, attention has recently been focused on shoot tip grafting as a possible solution to the problem of virus elimination without excessive rejuvenation.

Tissue culture has already proved effective in obtaining hybrid *Poncirus* plantlets from polyembryonic *Citrus* cultivars in which their development is usually inhibited. In these cases *Poncirus* pollen was used as a morphological marker. This pollen parent appears to promote strongly the development of hybrid embryos, while *Citrus* pollen does not. Thus, in order to secure the continued development of *Citrus* × *Citrus* hybrid embryos, it may be necessary to excise and culture all embryos at an early (e.g. heart-shaped) stage of development. Since such hybrids would be morphologically identical to nucellar plantlets, their early identification by some biochemical means would be highly desirable.

Mutation breeding of *Citrus* holds great promise, particularly when single, embryogenic cells are used. As plants from at least one cultivar can be regenerated from single cells, attention must now be given to the development of suitable mutagenic treatments and mutant screening procedures. The production of haploids and somatic hybrids may also prove valuable in the search for new, improved scion and rootstock cultivars.

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References see page 207.

5. Applications of Tissue Culture in Forestry

J. M. BONGA

1. Introduction

The world demand for wood products is expected to rise sharply over the next few decades from 130 million metric tons in 1970 to twice that amount by 1985 (WAYMAN, 1973). To meet this growing demand there will be an increasing need for large numbers of trees of improved quality and shortened rotation. Present-day tree improvement practices offer only limited possibilities to achieve that goal, and new techniques that can be used in addition to the older ones are required. One new technique that shows promise is tissue culture, and its use in forestry should be considered.

Since a clear distinction between forest and nonforest (e.g. fruit and ornamental) trees is difficult, and since the problems of propagation and genetic improvement are largely the same in both groups, tissue culture of both forest and nonforest species will be discussed. This review will deal primarily with those aspects of tree tissue culture that are most likely to lead to practical application in the near future. For coverage of other aspects of tree tissue culture, the reader is referred to recent reviews by DURZAN and CAMPBELL (1974a), WINTON (1972a, b, 1974a), and KONAR and NAGMANI (1974). To distinguish tree species from herbacious plants, Forestry Abstracts and the lists published by WINTON (1972a, b, 1974a) were followed.

2. Vegetative Propagation

Whenever vegetative propagation is economically possible, it is generally preferred to sexual propagation because genetic characteristics are better maintained (THULIN and FAULDS, 1968; MURASHIGE, 1974). The common methods of vegetative propagation are rooting of cuttings and grafting, the latter generally being used where rooting of cuttings has not been successful. However, rooting of cuttings is far from universally applicable and the use of tissue culture as a supplementary technique should be seriously considered.

2.1 Callus and Suspension Cultures

GAUTHERET (1934, 1937) was the first to obtain calli but not subcultures from cambial explants of angiosperm and conifer trees. Some tracheid formation was noted in these cultures and a few of the hardwood tree explants produced small

shoots (GAUTHERET, 1940a, b). The first regularly subcultured angiosperm tree callus cultures were those from *Salix caprea* (GAUTHERET, 1948), *Syringa vulgaris*, *Crataegus monogyna* (MOREL, 1948), and *Castanea vesca* (JACQUIOT, 1950), with some of the calli forming a few small shoots or roots (JACQUIOT, 1966). The first gymnosperm maintained successfully in continuous culture was *Sequoia sempervirens* (BALL, 1950). Some of its subcultured calli showed internal organization and a few produced buds. Continuous cultures of other gymnosperms soon followed (WINTON, 1972a; DURZAN and CAMPBELL, 1974a), especially after WHITE and RISSER (1964) and RISSER and WHITE (1964) established in detail most of the basic nutritional and physical parameters necessary for optimal growth of normal and tumor tissue of *Picea glauca*.

Recently, callus cultures of many tree species have formed plantlets, some of which have been transferred to soil and have grown into normal trees. Only rarely does this development proceed on just one nutrient medium and under one set of environmental conditions. Three main stages have been recognized (MURASHIGE, 1974), (1) establishment of a continuous culture, (2) morphogenesis leading to plantlet formation, and (3) transfer of plantlets from aseptic culture to soil.

In the early years of tree tissue culture, most cultures were started from explants containing interfascicular cambium and adjacent areas (called cambial explants) taken from the branches or the trunk of the tree. The techniques for obtaining such explants were originally developed by GAUTHERET (1934) and have been reviewed in detail by GAUTHERET (1959) and WHITE (1963). Adaptations of these techniques specifically for conifer cambial explants have been described by GRASHAM and HARVEY (1970).

The early popularity of cambial explants of woody species was due to their high content of endogenous cytokinin. This meant that callus could often be induced and maintained through several subcultures on a nutrient medium with an auxin as the only growth hormone (NITSCH, 1963a), which was a distinct advantage in the days before cytokinins were known as cell division regulators. Cambial explants of some tree species can initiate callus not only without cytokinin but also without auxin and some even without vitamins and amino acids in the culture medium. Callus of *Castanea vesca* and *Betula verrucosa* for instance, has been initiated and maintained, though not at optimal growth rate, through many subcultures on a medium containing only glucose or sucrose and mineral nutrients (JACQUIOT, 1967, 1972). Another advantage of cambial explants, especially if taken from the trunk of a big tree (WHITE and RISSER, 1964; WHITE, 1967) is that many replicates can be obtained from one large area of bark thus reducing morphological and physiological variation among the explants. However, most investigators prefer cambial explants from younger parts of the tree. STARITSKY (1970a) for example, noted that in *Coffea* spp. only the soft green internodes produced fast-growing callus. Similarly, WINTON and MATHES (1973) routinely used cambial explants from succulent juvenile shoots, and VENVERLOO (1973) and CHALUPA (1974) from young internodes of *Populus*. HRICOVA *et al.* (1974) used twigs and young stems of *Picea excelsa*, and HUHTINEN and YAHYAOGLU (1974) used twigs from young and mature *Betula pendula*.

The cambium and phloem tissues of the trunk and branches however, are not the only parts of the tree suitable for the induction of callus. In *Prunus amygdalus*

(MEHRA and MEHRA, 1974) and *Tilia americana* (BARKER, 1969) no differences in growth rates and morphology were detected in calli from explants from various parts of the tree.

Callus initiation is often affected by season. SUSSEX and CLUTTER (1959), using explants of several gymnosperm and angiosperm trees, found best proliferation when the explants were collected in the spring. BYTCHENKOVA (1963) reported similar results except that generally callus growth was poor shortly after bud-break. TRIPPI (1963) studied callus proliferation from internode explants of juvenile and adult branches of *Robinia pseudoacacia* and *Castanea vulgaris* and found no seasonal effect in explants from the juvenile zone of the tree; those from the adult zone proliferated less if collected during the flowering season. GRASHAM and HARVEY (1970) reported a delayed callus development from conifer explants collected in mid winter.

Most tree callus cultures are grown at a constant temperature and generally grow well within a wide range around the optimal temperature (WHITE and RISSER, 1964), although some cultures are temperature sensitive (STARITSKY, 1970a). Pine callus cultures occasionally go dormant if continuously exposed to optimal constant temperature (BONGA, unpublished). Placing the cultures in cold storage for about 6 weeks will break this dormancy and rapid growth will resume when the cultures are returned to higher temperatures. There is only one detailed study of the effect of different light regimes on tree callus growth (WHITE and RISSER, 1964). It showed little difference in growth between *Picea glauca* callus grown in darkness and various photoperiods at different intensities.

The second of the three stages of regeneration, morphogenesis leading to plantlet formation, is much more difficult to achieve than the induction and continuous culture of callus. The high auxin concentrations commonly used for callus induction and maintenance are generally inhibitory to morphogenesis and in most cases have to be replaced by a precise auxin-cytokinin balance to obtain proper root and shoot primordia. Originally kinetin was the commonly used cytokinin but now it is often replaced by others, especially benzylaminopurine (JACQUIOT, 1968; WINTON, 1971; CHALUPA, 1974; CAMPBELL and DURZAN, 1975). However in spite of extensive efforts, varying the auxin-cytokinin ratio does not always lead to morphogenesis. For example, LEE and DE FOSSARD (1974) tested 175 different auxin-cytokinin combinations on callus cultures of *Eucalyptus bancroftii* with no distinctive morphogenetic response.

Many chemical factors other than auxins and cytokinins have been found to affect morphogenesis (MURASHIGE, 1974). Bud formation in callus of *Ulmus campestris* depended on the proper balance of auxin and meso-inositol (JACQUIOT, 1951, 1966). Abscisic acid which is naturally present in conifers (LITTLE *et al.*, 1972) and angiosperm trees (WAREING and RYBACK, 1970) generally acts as an inhibitor, though it sometimes has a stimulatory and often a distinct morphogenetic effect. For instance, it assured normal development of embryos of *Taxus baccata* in in vitro culture (LE PAGE-DEGIVRY, 1973), induced rooting (BASU *et al.*, 1970) and stimulated callus formation in cultures of axillary buds of *Citrus sinensis* (ALTMAN and GOREN, 1971). ISIKAWA (1974) observed that shoot induction in hypocotyl cultures of the conifer *Cryptomeria japonica* was stimulated by

abscisic acid. DUTCHER and POWELL (1972) found that it inhibited elongation of apple buds in culture.

When establishing callus cultures, one tends to select experimental conditions that give a maximum growth rate. Under such conditions however, the cells are generally kept in a more or less permanently dedifferentiated state. Abscisic acid may act morphogenetically, in part, by slowing down the growth rate. Slowing of the growth rate by other means, for instance by sucrose starvation and aging of the cultures, has stimulated embryo formation in cultures of *Cryptomeria sinensis* (KOCHBA *et al.*, 1974).

One of the most important factors in obtaining morphogenesis is choice of explant. Although as was pointed out earlier, equally fast growing calli are often obtained from explants from different parts of the tree (BARKER, 1969; MEHRA and MEHRA, 1974), the morphogenetic potential of these calli often varies with the origin of the explant. In cambial region explants of *Picea glauca* and presumably in cambial explants of other trees, callus arises first mainly from the cambial cells; a few cells arise later from phloem parenchyma and xylem parenchyma cells (WHITE, 1967). The cambium is a secondary meristem and many of its secondary characteristics, such as large vacuoles, are carried over into the callus and subsequent subcultures (GAUTHERET, 1966; JACQUIOT, 1967, 1972). Secondary type vascular tissues differentiated readily in calli from cambial explants of several tree species but primary vascular tissues remained absent (JACQUIOT, 1967, 1972). This does not necessarily mean that calli derived from cambial cells lack totipotency. On the contrary, WINTON (1970) obtained *Populus tremuloides* and HUHTINEN and YAHYAOGLU (1974) *Betula pendula* trees from cambial explants. Presumably, it merely indicates that in those cases where the callus forms only secondary structures, complete dedifferentiation, a necessary condition before totipotency can be expressed (ZEEVAART, 1966; HALPERIN, 1970), was not obtained. In cultures of some species it may be very difficult to achieve complete dedifferentiation and in this case it is advisable to try explants from various parts of the plant in the hope of finding explants that contain a large proportion of morphogenetic cells, i.e. cells that are generally small, have small vacuoles and dense cytoplasm (HALPERIN, 1970; MCWILLIAM *et al.*, 1974).

This has led to morphogenesis in callus cultures of many tree species and in a few cases to regeneration of complete trees. ABBOTT and WHITELEY (1974) obtained callus from shoot tip meristem explants of apple and induced roots and shoots in this callus. In contrast, they also found that callus from stem explants, i.e. from cambial cells, failed to form roots and shoots. Shoots, which were later rooted and potted in soil, developed from callus from shoot tip explants of *Populus* × *canadensis* hybrid (BERBEE *et al.*, 1972). Roots, shoots, embryos, and sometimes plantlets large enough to be potted were obtained either directly or from callus from sections of embryos or very young seedlings of *Pinus* spp. (GREENWOOD and BERLYN, 1965; SOMMER *et al.*, 1975), *Picea glauca* (CAMPBELL and DURZAN, 1975), *Biota orientalis* (KONAR and OBEROI, 1965), *Gnetum ula* (VASIL, 1963), *Cryptomeria japonica* (ISIKAWA, 1974), *Ulmus americana* (DURZAN and LOPUSHANSKI, 1975), *Santalum album* (RAO and RANGASWAMY, 1971) olive (GILAD and LAVEE, 1974), *Citrus madurensis* (GRINBLAT, 1972), *Hevea brasiliensis* (MUZIK, 1956), *Ilex aquifolium* (HU and SUSSEX, 1971), and oil palm, *Elaeis guineensis*

(RABÉCHAULT *et al.*, 1970). From a tree improvement point of view, regeneration from sections of embryos and young seedlings has one distinct disadvantage. Though these embryos and seedlings may have been obtained from seeds of selected superior trees, their genotype will be different from that of the parent tree and the superior characteristics of the latter are not necessarily retained by the propagules.

A tissue with a very high morphogenetic potential is the nucellus of *Citrus*, which often forms adventive embryos naturally. This regenerative capacity of the nucellus varies between different cultivars and species and is inversely correlated with the amount of a specific anti-embryonic substance in the nucellus (MURASHIGE, 1974). Embryos, plantlets, and potted plants of several *Citrus* varieties were obtained from callus of nucellar origin (RANGAN *et al.*, 1969; BITTERS *et al.*, 1972; KOCHBA *et al.*, 1972). Callus from the ovules and unfertilized ovaries was equally morphogenetic (MITRA and CHATURVEDI, 1972; KOCHBA and SPIEGEL-ROY, 1973). In contrast, ovary wall tissues of many other fruit trees produced rapidly proliferating callus that did not regenerate plantlets (NITSCH, 1963b).

Immature pollen in many herbacious species is highly embryogenic and viable plants have been obtained from such pollen in anther cultures (NITSCH, 1972) (see Chap. II. of this Vol.). Limited morphogenesis has been obtained in callus derived from pollen of the following gymnosperms: *Ginkgo biloba* (TULECKE, 1965), *Taxus brevifolia* and *Taxus baccata* (TULECKE, 1959; ROHR, 1973), *Torreya nucifera* (TULECKE and SEHGAL, 1963), *Ephedra foliate* (KONAR, 1963), and *Pinus resinosa* (BONGA, 1974b). Callus from pollen of angiosperm trees has been reported for *Prunus persica* and *Prunus amygdalus* (MICHELLON et al., 1974), and callus and plantlet formation for *Populus* spp. (SATO, 1974). In *Hevea brasiliensis* anther cultures, the diploid connective tissues and filament produced morphogenetic callus (PARANJOTHY, 1974). On the other hand, callus from anther filaments of *Citrus* lacked morphogenetic potential (KOCHBA and SPIEGEL-ROY, 1973).

Root-forming callus was obtained from the haploid endosperm (megagametophyte) of *Picea abies* (HUHTINEN, 1972) and root- and shoot-forming callus from the haploid endosperm of the gymnosperms *G. biloba* (TULECKE, 1965), *Zamia integrifolia*, and *Cycas circinalis* (NORSTOG, 1965; NORSTOG and RHAMSTINE, 1967), and *Picca abies* (BONGA unpublished, Fig. 1). Not only the haploid endosperm of the gymnosperms but also the triploid endosperm of some angiosperm tree species has been capable of morphogenesis. Triploid plantlets were formed in endosperm callus cultures of *Putranjiva roxburghii* (SRIVASTAVA, 1973) and organogenesis occurred in endosperm callus of *Jatropha panduraefolia* and *Leptomeria acida* (JOHRI and SRIVASTAVA, 1973).

Morphogenetic callus has also been obtained from flower primordia. BAWA and STETTLER (1972) cultured female catkin primordia of black cottonwood tree, *Populus trichocarpa*. Some of the primordia developed female flowers, others formed callus with roots and leafy shoots. STRAUS and EPP (1960) cultured staminate cones of *Cupressus funebris* but the callus from these did not differentiate. Inflorescences have also been used to propagate coconut and oil palms. The palm trees are one plant group that urgently requires improved vegetative propagation techniques. Propagation and breeding of these economically important trees are unusually difficult because they are propagated mainly from seeds, but seed pro-

duction is low and germination may take several years (BOUVINET and RABÉCHAULT, 1965; STARITSKY, 1970b; SCHWABE, 1973). They form only a few leaves each year and, therefore, only a few vegetative apices are available for propagation. On the other hand, they flower abundantly and consequently attempts are now being made to change flower primordia, cultured in vitro, into vegetative shoots which can then be rooted (SCHWABE, 1973; BLAKE *et al.*, 1974). A different approach to propagation, the production of morphogenetic callus directly from inflorescences and embryos has already been accomplished in cultures of the oil palm *Elaeis guineensis* (RABÉCHAULT *et al.*, 1970; BARRET and JONES, 1974).

Lignotubers from *Eucalyptus citriodora* formed callus which produced plantlets, while callus from root tips and stem segments formed only callus (ANEJA and ATAL, 1969). LEE and DE FOSSARD (1974), however, found the callus from both stem segments and lignotubers of *Eucalyptus bancroftii* to be unresponsive.

In the foregoing, many examples of the formation of small plantlets in tree callus cultures have been cited. Why have so many of these plantlets failed to grow large enough for transfer to soil? Most authors do not discuss the possible reasons for this failure. However, it deserves close attention because some basic problems are involved and the step from small plantlet to viable potted plant may in many cases be a major hurdle. Few authors give details of the morphological structure of the plantlets. JACQUIOT (1964) reported the lack of secondary structures in roots and a much delayed formation of such structures in shoots arising from callus cultures of *Populus tremula*, *B. verrucosa*, *Tilia parvifolia*, *Ulmus campestris* and *Quercus sessiflora*. Similarly, the roots originating from haploid callus from megagametophytes of *Picea abies* (HUHTINEN, 1972; BONGA unpublished; Fig. 1) lacked secondary vascular systems. The induction of vascular elements probably takes place in two stages, the first, the formation of provascular cells, requires cytokinin, the second stage primarily requires other factors (MINOCHA and HALPERIN, 1974). Apparently, in many plantlets the second stage did not materialize and presumably this was one reason for the failure of further growth.

A second reason why many of the root and shoot producing calli did not form viable plants was the failure to establish functional vascular connections through the callus between the shoots and roots. HUHTINEN and YAHYAOGLU (1974) noticed a lack of vascular connections between roots and shoots formed on callus of *B. pendula*. Vascular connections develop under the directing influence of active buds as was shown by grafting buds on callus of *Syringa vulgaris* (WETMORE and SOROKIN, 1955; WETMORE and RIER, 1963) and *Castanea vesca* (JACQUIOT, 1972). Therefore, failure to from vascular connections between roots and shoots in callus cultures probably is often due to insufficient shoot activity. Directional vascular tissues have been artificially induced in callus of *Fraxinus americana* and *Syringa vulgaris* by topical application of auxins and sucrose (WETMORE and RIER, 1963). Organization of vascular tissues depends also on physical factors. BROWN (1964) induced vascular tissues in *Populus deltoides* cambial explants by pressure, and DOLEY and LEYTON (1970) in callus of *Fraxinus excelsior* by water stress.

Transfer of plantlets from the culture medium to soil is often a difficult procedure although sometimes the transfer can be made without much difficulty as was the case with *Populus tremuloides* and *P. tremula* (WINTON, 1970, 1971) and *B. pendula* (HUHTINEN and YAHYAOGLU, 1974). A gradual adaptation to the soil environ-

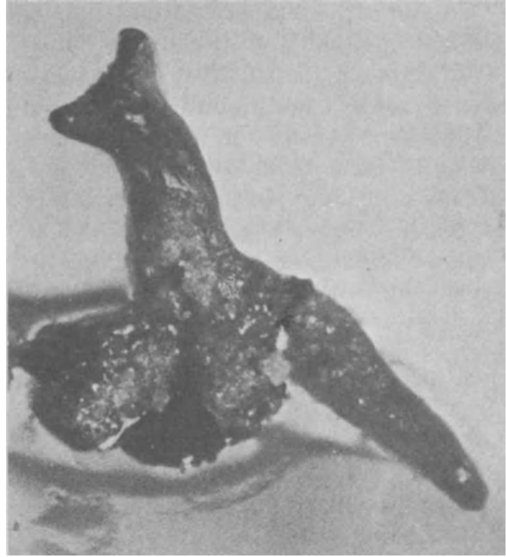

Fig. 1. A plantlet originating from callus from a piece of megagametophyte tissue dissected from a mature seed of *Picea abies* L. Karst. It was cultured on BROWN and LAWRENCE'S (1968) medium with 0.2 mg/l indoleacetic acid instead of 2,4-dichlorophenoxyacetic acid. Chromosome counts of callus and root tip cells showed that these tissues were haploid (BONGA, unpublished)

ment is usually required, involving a gradual simplification of the nutrient medium and increased light intensities (MURASHIGE, 1974). Water stress is often a problem and high atmospheric humidities have to be provided (WINTON, 1970; BITTERS *et al.*, 1972). It has been noted that roots penetrating the nutrient medium often lack root hairs. For example, the roots of plantlets of *Populus* spp. had to be kept above the nutrient surface for root hairs to develop and for the roots to become functional (WINTON, 1970; CHALUPA, 1974).

One major problem in propagation from callus cultures is genotypic variation between the propagules, resulting from genetic instability of the callus cultures (SUNDERLAND, 1973; MURASHIGE, 1974, Chap. III.1 of this Vol.). How serious this is in the propagation of trees from callus cultures is not yet known, because the number of trees thus propagated is still too small for a proper assessment. Chromosome abnormalities are common in cultures of gymnosperms. Sticky and clumping chromosomes, micronuclei, and abnormal nuclei have been found in callus cultures of *Pinus strobus*, *P. densiflora* and *P. resinosa* (GAUTHERET, 1956; TOMINAGA and OGA, 1970; BONGA, 1974b). Polyploid and aneuploid cells were found in cultures of *G. biloba* (TULECKE, 1957) and *Picea glauca* (DE TOROK and RODERICK, 1962; DE TOROK, 1968). PARTANEN (1963) found a few tetraploid cells in cultures of *Pinus lambertiana.* Cells with less than the haploid number of chromosomes appeared in cultures of tumors of *Picea glauca* (DE TOROK and WHITE, 1960) and microsporophylls of *Pinus resinosa* (BONGA, 1974b). With regard to cultures of angiosperm tree species, WRIGHT and NORTHCOTE (1973) found old cultures of *Acer pseudoplatanus* to be tetraploid. MEHRA and MEHRA (1974) noticed diploid, triploid, tetraploid, and aneuploid cells in callus cultures of *Prunus amygdalus*, with the number of polyploid and aneuploid cells increasing after several subcultures.

Abnormalities in chromosome number and structure may not be the sole cause of variation in propagules. Morphogenetic expression is also influenced by cytoplasmic, epigenetic factors (ZEEVAART, 1966; HALPERIN, 1970) and variation in such factors could result in variation in propagules. The significance of cytoplasmic genetic factors in processes such as cell growth, rootability, graftability, juvenility, and aging in forest tree species has been reviewed by STANLEY (1970). The following examples are suggestive of epigenetic controls in cultures of woody species. In callus cultures of *Sequoia sempervirens* files of tannined cells arose from tannined initials and persisted through many subcultures (BALL, 1950). STOUTEMEYER and BRITT (1965) observed that callus derived from juvenile *Hedera helix* consistently behaved differently from callus derived from mature plants, the differences being maintained through many subcultures. TULECKE (1967) has described plastid transformation and replication and its effects on cultures of *Ginkgo biloba*.

2.2 Bud Cultures

A clear distinction should be made between bud cultures and apical meristem cultures. Cultures of the apical meristem ideally involve only the actual apical dome of the shoot, sometimes with, though preferably without a few leaf primordia attached. They are often used to obtain disease free clones (SMITH and MURASHIGE, 1970) and have been used in studies of the mechanisms of vegetative and reproductive growth of *Picea abies* (ROMBERGER, 1966; ROMBERGER and TABOR, 1971). Bud cultures on the other hand, involve the entire rudimentary vegetative shoot collected either just before budbreak or during the preceding dormant season. Propagation is achieved by rooting of the buds or of short node sections with axillary buds. The advantage of this method over propagation by callus or suspension culture is that a shoot apex is a priori present and does not have to be induced, only root induction being required. The main disadvantage is that one explant will form only one propagule, not the hundreds or thousands that are sometimes produced in callus and suspension cultures. Since the literature dealing with propagation of trees by bud culture has been reviewed recently (BONGA, 1974a), the present discussion will be limited chiefly to literature not discussed at that time.

Little growth was obtained in cultures of axillary buds of several hardwood species (BEAUCHESNE, 1966). Shoot elongation but no rooting occurred in bud cultures of *Acer platanoides* (DEMAGGIO and FREEBERG, 1969) and *Malus sylvestris* (ELLIOT, 1972). Successful rooting of *Malus* buds was reported by DUTCHER and POWELL (1972) and WALKEY (1972). Buds of *Elaeis guineensis* were rooted by STARITSKY (1970b), those of *Prunus* by QUOIRIN *et al.* (1974).

Rooting depends on the complex interactions of stimulating and inhibiting substances in the buds as well as in the growing roots, and these substances have to be properly manipulated before successful rooting can be expected (BONGA, 1974a; HAISSIG, 1974a, b). In particular, dormancy is an aspect that requires consideration. Although dormant buds of some tree species elongated readily in culture (AL-TALIB and TORREY, 1959, 1961; CHALUPA and DURZAN, 1973), buds

of other species showed a seasonal trend (ALTMAN and GOREN, 1974). Dormancy in buds is not always followed by nondormancy in a simple two-phase annual cycle. BACHELARD and WIGHTMAN (1974) found two peaks in inhibitor over stimulator ratios in buds of *Populus balsamifera*, one in early winter, the other just before budbreak. Inhibitors can often be removed from tissues simply by washing in water. GOOD (1974) for example, describes the leaching of an inhibitor resembling abscisic acid from the foliage of *Picea sitchensis* and *Betula pendula*. Soaking in water was a prerequisite for successful rooting of nodes of *Eucalyptus grandis* in culture (CRESSWELL and NITSCH, 1975). Using conventional rooting of cutting techniques this species can be propagated only from juvenile nodes up to node 14 but was propagated in tissue culture from nodes up to node 50.

3. Tree Improvement

Several analyses of the economics of tree improvement programs have been carried out and have led to the consensus that such programs generally are well worth the cost. Even with yield increases as low as 2–5% the financial benefits could be significant (LIBBY *et al.*, 1969; CARLISLE and TEICH, 1971). Therefore, a discussion of the potential value of tissue culture to tree breeding is of more than just academic interest.

3.1 Haploid Tissue Cultures

For most tree species inbreeding to obtain homozygous lines for controlled hybridization is not practical because of the long period between successive generations. This problem is aggravated by the fact that most forest trees, at least those in temperate climate zones, are highly heterozygous and inbreeding often leads to low seed set and reduced seedling growth (BAKER, 1959; LIBBY *et al.*, 1969; WINTON and STETTLER, 1974). Furthermore, many tree species are dioecious and therefore cannot be self pollinated. To circumvent some of these problems, a technique now frequently considered is doubling of the chromosome number in either naturally occurring or artificially obtained haploid plants. Haploid trees have been produced artificially by remote hybridization, pollen irradiation, and chemical treatment of pollen (STETTLER and BAWA, 1971; WINTON and STETTLER, 1974).

Lately viable haploid plants have been obtained from anther cultures of several agricultural species (SUNDERLAND, 1973; Chap. II. of this Vol.), but as yet not from tree species. Haploid tissue cultures of trees have so far been restricted mainly to the gymnosperms, with haploid callus arising from pollen and haploid megagametophyte (endosperm) tissues. The early work with haploid gymnosperm tissues has been reviewed by TULECKE (1965); later, haploid cultures were obtained in *Pinus* spp. (BORCHERT, 1968; BONGA, 1974b), *Taxus baccata* (ZENKTELER and GUZOWSKA, 1970; ROHR, 1973), *Juniperus* spp. (DUHOUX and NORREEL, 1974),

and *Picea abies* (Huhtinen, 1972). Of the angiosperm tree species, anther cultures of *Prunus persica* and *P. amygdalus* have formed callus with haploid, diploid, and triploid cells (Michellon *et al.*, 1974), and those of *Prunus avium* multicellular pollen (Jordan, 1974). The formation of callus from pollen is sometimes stimulated by cold treatment (Bonga, 1974b) or centrifugation (Bonga and McInnis, 1975) of the material just before transfer to the culture medium.

There are several complications associated with haploid cultures that require discussion. First, the pollen in an anther or microsporophyll have various genomes. Consequently, mixing the cell masses that arise from several pollen will result in a nonisogenic callus and plants derived from such a callus would be chimeras (Sharp *et al.*, 1972). Therefore, this method is only applicable if plants are regenerated from single cells of such a callus or if embryos develop directly from individual pollen. Fortunately, with gymnosperms we do not depend on pollen as a source of haploid cells. The megagametophyte provides a considerable mass of tissue and because all cells have originated from one megaspore, they all have the same haploid genome.

A second problem in producing homozygous diploids from haploid cultures is the presence of "deleterious recessive genes". Many tree species carry a heavy load of deleterious recessive genes and growth depression can be expected in the artificially produced homozygous diploid plants of these species (Winton and Stettler, 1974).

In cultures of haploid tissues of gymnosperms, limited differentiation is common (LaRue, 1954; Radforth and Bonga, 1960; Bonga, 1974b). The most advanced stage of differentiation, small plantlets, have occurred in cultures of megagametophytes of *Zamia integrifolia* (Norstog, 1965) and *G. biloba* (Tulecke, 1965). To what extent the lack of further organized growth should be attributed to deleterious recessive genes or to limitations imposed by the culture techniques is not known.

There are other haploid tissues besides pollen and megagametophytes that could be used to start haploid cultures. In addition to embryos induced by haploid parthenogenesis (Stettler and Bawa, 1971), naturally occurring reverse and polyembryos are often haploid (Yakovlev, 1967; Ching and Simak, 1971; Huhtinen, 1972). Polyembryos appear most frequently in seeds of trees in arctic climates and can easily be detected by X-ray radiography (Simak, 1973). Usually they do not germinate because of competition from the normal embryos but by dissection and transfer to a culture medium they could be made to grow.

3.2 Polyploid Tissue Cultures

Polyploidy is common in agricultural crops, less common in angiosperm forest trees and rare in gymnosperms (Gustafsson, 1960; Stanley, 1970). Tree breeders are interested in polyploids because in some genera, for example *Populus* (Gustafsson, 1960; Muhle Larsen, 1970), the polyploids show higher growth rates than the diploids, but in other genera (Libby *et al.*, 1969) they are weak.

In the course of evolution polyploidy has played a major role in the creation of new species, primarily through remote hybridization (allopolyploidy) (Baker,

1959; STEBBINS, 1971). In contrast to allopolyploidy, autopolyploidy, which results not from hybridization but from direct doubling of chromosome sets, generally leads to weak and sterile polyploid plants. Therefore, artificial chromosome doubling is often ineffective as a breeding tool for trees (STANLEY, 1970; WINTON, 1974b) and also may hinder regeneration of trees from callus cultures.

The naturally evolved polyploids among long-lived species often have a low fertility. Natural polyploidy, therefore, is rare among trees that do not propagate vegetatively (BAKER, 1959). It can be expected that a fair proportion of polyploid trees, newly developed from tissue cultures or by other methods, also will show various degrees of sterility. They would, therefore, only be of economic use if means of vegetative propagation were available. Tissue culture could be of considerable value in that respect. In fact, callus cultures are already being used on that basis in the propagation of triploid and tetraploid *Populus* (WINTON, 1970, 1971; WINTON and EINSPAHR, 1970).

The following is a brief review of the occurrence and behavior of polyploid cells in tree tissue cultures. DE TOROK (1968) established through single cell isolations, stable $1n$, $2n$, $3n$, etc. up to $14n$ cell lines of tumor tissues, and up to $5n$ of normal tissues of *Picea glauca*, with the growth rate increasing with the ploidy level. High ploidy obviously is not a deterrent to rapid division and growth in relatively unorganized cell systems. Very high ploidy levels (BAYLISS and GOULD, 1974) and tetraploid cells (REMBUR, 1974) were also detected in cultures of *Acer pseudoplatanus*. WRIGHT and NORTHCOTE (1973) established diploid as well as predominantly tetraploid cell lines in cultures of the same species, with only the diploid cell lines having the capacity to form roots. MEHRA and MEHRA (1974) found diploid, triploid, and tetraploid cells in callus cultures of *Prunus amygdalus*, but only the diploid cells participated in the regeneration of plants. However, a ploidy up to the tetraploid level in *Populus* (WINTON, 1970, 1971) and triploid level in *Putrenjiva roxburghii* (SRIVASTAVA, 1973) callus did not interfere with regeneration of plantlets.

3.3 Embryo Cultures

One of the oldest applications of tissue culture in plant breeding is embryo culture (see Chap. III.3 of this Vol.). The main objective of this technique is to germinate embryos that would not normally germinate because of incompatibility between the embryo and maternal tissues, as is common in seeds resulting from interspecific hybridization (HAGMAN, 1972).

MAHESHWARI and RANGASWAMY (1965) have reviewed the extensive literature dealing with the culture of embryos of interspecific crosses in fruit trees. Trees have been raised from hybrid embryos of plum, apple, peach and pear. Embryo culture has also helped to shorten the breeding cycle and has hastened germination of hybrid and nonhybrid embryos of apple, almond, plum, peach and cherry that normally are slow to germinate. It has also been used to accelerate germination of oil palm seeds. These often take several years to germinate under natural conditions, but their embryos, if excised and cultured in vitro, will germinate within a few days (BOUVINET and RABÉCHAULT, 1965). Similarly, dissected em-

bryos and immature ovules of *Hevea brasiliensis* have been cultured to obtain improved germination (MUZIK, 1956).

In efforts to obtain virus resistant papaya, NAGAI (1974) isolated hybrid embryos and raised plantlets from these on a culture medium. In *Citrus* the development of hybrid embryos is often inhibited by the spontaneous growth of adventitious polyembryos arising from the nucellus and therefore carrying the maternal genotype. By dissection and in vitro culture of the desired hybrid embryo, RANGAN *et al.* (1969) obtained vigorous hybrid plants. STONE and DUFFIELD (1950) reared plants from hybrid embryos of *Pinus*, by placing ovules from which the seed coat and nucellar tissues had been removed aseptically, on just agar with water.

Another technique for obtaining otherwise incompatible hybrids is by in vitro pollination followed by embryo culture. It is used where hybridization fails because of incompatibility between pollen and pistil (MAHESHWARI and RANGASWAMY, 1965; KAPIL, 1967).

3.4 Protoplast Hybridization

Protoplast hybridization is a technique in which naked protoplasts of somatic or gametic cells of different plants are fused, and plants are raised from the fused protoplasts (see Chap. IV.1 of this Vol.). This has the potential for controlled hybridization of plants, including trees. By fusing haploid protoplasts from two different trees, followed by regeneration of cell walls around the fused protoplasts, cell division, and morphogenesis, one should be able to form new diploid hybrids (WINTON, 1974b; WINTON and STETTLER, 1974). Since the normal sexual mechanism and consequently some of the sexual barriers are bypassed, a wider range of hybridization is potentially possible; compatibility of the two genomes fused together being the limiting factor. Fusion of protoplasts of various ploidy should be considered as a means of producing autopolyploid or allopolyploid trees, again the main limiting factor being compatibility of the gene combinations. Protoplast techniques also allow introduction of foreign extrachromosomal genetic units, self-replicating organelles, plasmagenes, etc., thus increasing genetic variability. For a detailed discussion of these aspects and other applications of protoplast culture for tree improvement, the reader is referred to the reviews by DURZAN and CAMPBELL (1974a, b) and WINTON (1974b).

3.5 Mutant Selection

Presently mutants of tree species are obtained mainly by irradiation or application of mutagenic chemicals to pollen, seeds, or whole plants (PRIVALOV, 1972; DURZAN and CAMPBELL, 1974a, b). However, these methods are not always practical because mutation rates are often low, necessitating the use of large populations for effective mutant screening. This favors the use of cell suspension and subsequent cell plating techniques, because it allows easy handling of large populations of cells and embryos (DURZAN and CAMPBELL, 1974a, b; MELCHERS, 1974).

Although such techniques are now frequently employed to obtain mutant cell lines of herbacious plants, they have not yet been applied on a wide scale for selection of such lines of trees.

Auxin autotroph mutant cell lines were separated from suspension cultures of *Acer pseudoplatanus* treated with physical and chemical mutagens (LESCURE, 1969). SPIEGEL-ROY and KOCHBA (1973) found that gamma irradiation of ovular callus cultures of *Citrus sinensis* stimulated embryo formation but the effect appeared to be caused mainly by radiation-induced chemical changes in the nutrient medium. An arginine-requiring mutant cell line, originating from a callus obtained from *Ginkgo biloba* pollen, has been described by TULECKE (1960).

3.6 Gene Pool Preservation

Impoverishment of the natural gene pool is a problem of great concern to tree breeders in many countries. Even a country as rich in forest resources as Canada is facing serious gene losses in populations of some valuable tree species (FAO, 1974). As is pointed out by BAJAJ and REINERT (Chap. VII.3 of this Vol.) tissue culture could have an important function in the establishment of gene banks by using deep freeze storage techniques, particularly for plants that normally are propagated vegetatively, i.e. those that are infertile and therefore cannot be stored as seeds. With regard to trees, cells of *Acer pseudoplatanus* have been stored in liquid nitrogen and have resumed growth after thawing (SUGAWARA and SAKAI, 1974).

4. Control of Tree Diseases

Active meristems are often free of pathogens. By dissecting meristems from infected plants, by culturing these meristems, and by regenerating new plants from these cultures, pathogen-free plants are often obtained (see Chap. V. of this Vol.). This technique is particularly useful where the disease is carried from one generation to the next through pollen or seeds. Pathogen-free clones have been obtained in this manner for several species of trees. Shoot meristems have been used to produce virus-free cassava, *Manihot utilissima* (BERBEE *et al.*, 1973; KARTHA and GAMBORG, 1975), and *Populus* (BERBEE *et al.*, 1972); the disease free polar thus obtained, grew twice as fast as the diseased parent (ANON., 1974a). Virus-free *Citrus* plants were obtained from nucellar tissues in culture (BITTERS *et al.*, 1972).

Failure to culture obligate fungal parasites separate from their host has prompted the use of tissue cultures to study these organisms. CUTTER (1959) maintained callus of *Juniperus virginia*, systemically infected with *Gymnosporangium juniperi* through many subcultures, finally isolating the fungus on the artificial medium. *Pinus monticola* callus infected with *Cronartium ribicola* was cultured by HARVEY and GRASHAM (1969). They also grew the parasite physically separated from the host callus by a cellulose membrane (HARVEY and GRASHAM, 1970) and found that a callus from a nonhost, *Pseudotsuga menziesii*, could provide the required nutrients equally well (HARVEY and GRASHAM, 1971). SCHNEI-

DER and REVERDY (1973) studied the behavior of *Taphrina deformans* in infected callus of peach, and CHAUMONT *et al.* (1974) cultured conidia of *Podosphaera leucotricha* on callus of *Malus pumila.*

Studies have also been carried out with several of the angiosperm parasites of forest trees. The mistletoes are widespread throughout the world and cause considerable damage, the most destructive genus being *Arceuthobium*, the dwarf mistletoes. In many areas of western North America, *Arceuthobium* spp. is rated as the most serious of all pathogens in conifer stands (HAWKSWORTH, 1961; LEAPHART, 1963). Physical removal of infected trees or removal of infected parts by pruning will control the disease (LEAPHART, 1963). So far, biological as well as chemical control methods have been relatively ineffective (HAWKSWORTH, 1961). Herbicide sprays, though killing the small aerial shoots of the parasite, have no effect on the massive endophytic system, i.e. the part of the parasite that lives inside the host (HAWKSWORTH, 1961). To kill the endophytic system but not the surrounding host tissues requires a highly selective systemic herbicide and to find such a herbicide may be a difficult task (LEAPHART, 1963). The search for such specific herbicides would be made much easier if *Arceuthobium* could be grown separate from its host on a culture medium. This would allow separate studies of the physiology of the parasite and of the host. By capitalizing on physiological differences between host and parasite thus discovered, chemical control methods could be developed. Some of the other mistletoes, *Amyema*, *Dendrophthoe*, *Loranthus*, and *Phoradendron* are easier to control by chemical means than *Arceuthobium* (GILL and HAWKSWORTH, 1961), presumably because their shoots are much larger.

Many of the mistletoes have been grown in vitro. *Arceuthobium pusillum* was obtained from seeds in culture (BONGA, 1971, 1974c), several members of the Loranthaceae and *Phoradendron tomentosum* of the Viscaceae were cultured from embryos (BAJAJ, 1967, 1970) and several of the Loranthaceae and Santalaceae from mature endosperm (see Chap. III.4 of this Vol.).

Such cultures not only will set the stage for future investigations of chemical control measures of the parasites but they also will open the way to study penetration mechanisms, physical versus enzymatic (GILL and HAWKSWORTH, 1961), the nature of immune reactions of the host (SCHOLL, 1957; TAINTER and FRENCH, 1971), the cause of witches' brooming of the host (HAWKSWORTH, 1961), and other host-parasite interactions.

5. Secondary Products

Tissue cultures produce a large range of organic chemicals some of which are retained within the cells, while others are secreted into the nutrient medium (KRIKORIAN and STEWARD, 1969), or released into the atmosphere (STUART and STREET, 1971). The large-scale use of tissue culture in the industrial production of valuable organic products is now seriously being considered, especially for the pharmaceutical industry (see Chap. VI of this Vol.).

Valuable products often are not produced by the whole plant but in specialized tissues and organs. Therefore, attempts to increase production of these substances by tissue culture are frequently based on culture of these specialized tissues and organs. However, this specialization and the capacity to produce the desired compounds is often lost in culture, especially if cells are grown in the partially or completely dedifferentiated state prevailing in large-scale suspension cultures. For example, if lactifer cells which in situ produce latex rubber are cultured, the rubber producing capacity is largely lost (KRIKORIAN and STEWARD, 1969). On the other hand, cultured tissues sometimes synthesize rare and valuable chemicals that are not produced in appreciable amounts in tissues in situ (KRIKORIAN and STEWARD, 1969).

Tree tissue cultures have featured prominently in the search for methods to produce plant cells in large quantities. Mass production of *Grinko biloba* tissue has been described by NICKELL (1962), TULECKE and RUTNER (1965), and TULECKE *et al.* (1965), and of *Acer pseudoplatanus* by WILSON *et al.* (1971).

The following products of interest have been produced in tree tissue cultures. Amino acids in cultures of *G. biloba* (TULECKE and RUTNER, 1965), phenolics and tannins in cultures of *Juniperus communis* (CONSTABEL, 1965, 1968) and of *Pinus resinosa* (JORGENSEN and BALSILLIE, 1969), and antimicrobial agents in cultures of *Populus tremuloides* and *Phytolacca americana* (MATHES *et al.*, 1971; LIN and MATHES, 1973). Camptothecin, an antitumor and antileukemic alkaloid, was isolated from cultures of *Camptotheca acuminata* and potent plant virus inhibitors from cultures of *Pinus americana* (MISAWA *et al.*, 1974). Papain, an important proteolytic enzyme, was isolated from cultures of *Carica papaya* (MEDORA *et al.*, 1973). In most of these cases, however, only small amounts of the desired chemicals were extracted and their production on an industrial scale by tissue culture is still far from being realized.

6. Summary and Conclusions

Vegetative propagation by tissue culture has been achieved for several tree species. However, with the exception of *Citrus* which regenerated directly from callus, all were propagated either by rooting of shoots dissected from callus or by rooting of buds. These procedures however, are as cumbersome as conventional rooting of cuttings and can be classed as an improvement over the old methods only where it allows propagation of material that cannot be propagated conventionally, or where clone improvement (disease elimination, rejuvenation, etc.) is obtained. Besides vegetative propagation of some tree species and the elimination of pathogens by shoot apex or nucellus culture in others, the most notable practical application has been the introduction of new interspecific hybrids by embryo culture.

Considering the future prospects of tree tissue culture the following developments can be expected (1) Inbreeding followed by crossing of inbred lines to produce genetically improved plants, is generally considered impractical for trees

because of the long period between generations. Tissue culture may produce homozygous diploid plants rapidly by haploid culture, provided that problems associated with deleterious recessive genes and other genetic imbalances can be overcome. (2) Regeneration of new polyploid trees from polyploid cells in culture is a possibility, provided suitable genetic balances are obtained. (3) In cell cultures there is a continuously renewed range of either spontaneously occurring or mutagen induced genetic variation, some of which could be incorporated into new plants by regeneration from these cultures. (4) Somatic hybridization, once it has become a practical tool, will greatly enhance the possibility of recombining genetic as well as cytoplasmic factors. (5) To combat tree diseases, tissue culture could aid in the development of chemical control techniques. (6) Tree tissue cultures are already producing a number of interesting medicinal and other complex secondary products. This development has not yet reached commercial levels but some highly specific products undoubtedly will eventually be produced industrially from tree tissue cultures.

References see page 207.

6. Applications of Tissue Culture in the Improvement of Coffee

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1. Introduction

1.1 Major Coffee-growing Areas

The word coffee is used to characterize not only the plant but also the stimulatory drink obtained from the roasted and ground seeds of certain species of the genus *Coffea*, family Rubiaceae. The genus originally had a broad distribution in the tropical and subtropical parts of Africa and Asia. The stimulatory effects of roasted coffee beans were well known to the natives of Africa when the Arabs brought *Coffea arabica* seeds from Ethiopia to Yemen (Arabian Peninsula) during the 13th century, and established the first plantations. Its name seems to be derived from the arabic word *gahwah* although some authors try to associate its origin with Kaffa, a province in southwest Ethiopia where *Coffea* thrives spontaneously. At that time, coffee was known in Cairo and Mecca, and by the 14th century in Persia, and in Turkey by the 16th century (Sivetz and Foote, 1963). Following its introduction into Yemen, the cultivation of *Coffea* extended to Ceylon (1658), India (1695), Indonesia (1696), and to other regions with favorable crop conditions. Due to the great popularity of the beverage, its cultivation spread around the world, being introduced in Surinam in 1714, Haiti and Dominican Republic in 1715, Martinique 1723, Brazil 1727, Jamaica 1730, Puerto Rico 1755, Costa Rica 1779, Cuba 1784, Venezuela 1784, Mexico 1790, El Salvador 1852, Colombia late in the 18th century, and gradually to other countries.

In 1690, the Dutch brought seedlings from Yemen to Java and in 1706 a single seedling, *C. arabica* L. var *arabica* L., from Java was placed in the Botanical Garden in Amsterdam. This tree gave rise to the seeds used to develop coffee plantations in the Americas which accounts for the uniformity found among coffee trees in Central and South America (Carvalho, 1946).

The genus *Coffea* consists of at least 64 species which are grouped in four sections (Chevalier, 1947). Some economically important species are *Coffea arabica*, *C. canephora*, *C. liberica*, and *C. dewevrei*. All species of the genus *Coffea* so far examined have proven to be diploid, except *C. arabica* which is a tetraploid. During recent years interest has been placed on species of the section Paracoffea which are characterized by the production of caffeine-free beans. These species occur widely on the Madagascar Island (Leroy, 1962).

Coffea arabica is for the most part considered restricted to highlands and according to data obtained from the Pan-American Coffee Bureau (1973), it has been responsible for 70% of the total world coffee production during the years 1969 to 1973. It is practically the only species cultivated in Latin America.

Brazil, having 38% of this species on the continent, is the largest arabic coffee producer. Five other important countries producing *C. arabica* are Colombia, Mexico, El Salvador, Ethiopia and Guatemala.

C. canephora Pierre ex Froehner cv. Robusta, which contributes 26% of the total coffee production, is the second most important coffee species cultivated. It is found mainly in Africa and is highly polymorphic. The major countries cultivating Robusta are listed in the following order: Ivory Coast, Angola, Uganda, Indonesia, Zaire, and Cameroun (Pan-American Coffee Bureau, 1973).

C. liberica Bull ex Hiern and *C. dewevrei* DE WILD et are two other species of lesser importance cultivated for commercial purposes. The former is found almost exclusively in Malaya and Surinam, and to some extent in other countries such as Guinea, Ivory Coast, Liberia, Gabon and Angola as part of a mixed population of coffee species. *C. dewevrei* cv. Excelsa is mainly found in the Ivory Coast and Central African Republic (KRUG, 1963).

1.2 Economics

In the international trade market, coffee is one of the most important products. It is the most widespread permanent crop in the tropical regions of the world and is of decisive importance in the economics of developing countries. At present, about 7 million hectares are under coffee cultivation. Half of the total acreage of coffee is found in Latin America.

The size of coffee plantations varies from 100–200 trees to large plantations with several million trees, as in Brazil or Angola. Among the ten major coffee producers in the world during the past five years, Brazil leads with approximately 27% of the total production, followed by Colombia, Ivory Coast, Angola, Mexico, Uganda, Indonesia, Ethiopia, Guatemala, and El Salvador (Pan-American Coffee Bureau, 1973). In Table 1 the production and the revenue of these major coffee producers are listed according to the classification system adopted by the international trade market.

Based on the statistics of the Pan-American Coffee Bureau (1973), a total of 4.5 billion US dollars was generated by coffee in the international trade market during 1973. Brazil and Colombia, the two major producers, received together 43% of this revenue. An interesting comparison comes from the relative importance of coffee in the value of total trade budget of the producing countries. In four countries, coffee contributes more than one-half of the total exportations: Burundi (93%), Uganda (66.3%), Rwanda (61.7%), and El Salvador (52.1%). Among the six others, the importance of coffee revenue is more than one-fourth: Ethiopia (42.2%), Angola (39.0%), Colombia (39.3%), Haiti (35.5%), Guatemala (32.2%), and Costa Rica (30.4%). In Brazil, this figure is 21.7%. The pattern of distribution of coffee imports during 1973 (% of total imports) was according to the following: United States (37%), European Economic Community (34%), other western countries (13%), Eastern Europe (5%), Asia and Oceania (6.5%), and others (4.5%). The prices of coffee have undergone substantial fluctuation, hence resulting in altered world consumption and production. During the past 10 years (1964–1973) the world consumption increased at an average rate of 2%

Table 1. Classification and average of total production of coffee for the 10 major world producers from 1969 to 1973 and exportation revenues during 1973

Classification and country	Thousands of 60 kg bags	Millions of US dollars
Colombian Milds (arabica)		
Colombia	8350	598
Unwashed arabics		
Brazil	18170	1343
Ethiopia	2090	103
Other arabic milds		
Mexico	3355	117
El Salvador	2234	191
Guatemala	2044	145
Robusta		
Ivory Coast	4225	208
Angola	3450	199
Uganda	2990	228
Indonesia	2450	74
Others	28095	1294
World total	67453	4500

per year, while production increased by a fraction of 0.5%. Africa has been emerging in importance as a coffee producer from 1.3% of the world market prior to World War I to 27% of the world market in 1973 (KRUG, 1963; PAN-AMERICAN COFFEE BUREAU, 1973). It is apparent that this increased participation of the African countries in coffee exports has been a consequence of the relatively decreased importance of Brazil in the international coffee market. On the other hand, Colombia, the second major producer, has maintained its original relative position.

1.3 Coffee Industry

The excellence of the arabic coffee beverage explains why *C. arabica* cultivars predominate among the coffee producing countries. However, this species has two restrictions: a reduction of productivity and quality of the beverage in lowlands and the susceptibility of the most productive cultivars to the main fungal diseases (coffee berry disease, *Colletotrichum coffeanum*, and coffee leaf rust, *Hemileia vastatrix*). Of course, these diseases can be controlled by fungicides, but this is not always economically feasible. Disease resistance is an individual characteristic which was first used in *C. canephora*. At present, there are many *C. arabica* rust-resistant cultivars being used in breeding programs.

Robusta coffee has the advantage of lower cost in the international market and the supposed property of containing more soluble solids for the production of instant coffee. These two reasons partially explain why Robusta has shown gain on the trade market from 3% (1923–1924) to 25% (1961–1962). Coffee berries can

be harvested in two ways. (1) stripping the branches or (2) individual selection by hand picking. The first process is faster and less expensive, but has the disadvantage of carrying many impurities, e.g. green coffee, leaves, and pieces of wood. The latter mode of harvest selects only ripened berries and provides for superior preparation.

The preparation of the coffee beans can be made by a *dry* or *wet* process. The dry method gives the "unwashed" type of coffee whereby the berries are allowed to dry on the trees and then harvested by stripping the branches. The berries are washed, graded and dried to a water content of 12–15% on cement patios under the sun or in driers. Then the pericarp and the silver skin are removed and subsequently the beans are classified by size, shape and color. The normal berry gives two seeds which have a convex and a flat side. In the wet process, the ripened coffee berries are harvested by hand and then the exocarp is removed. This process gives the "washed" type of coffee. "Green coffee" is the commercial term for coffee prior to the roasting process. Classification and trade are performed using the green coffee.

The price of coffee depends primarily on species, arabics being more expensive than Robusta coffee, and better in quality. However, among the washed and unwashed arabics, the unwashed coffee has less commercial value.

There have been several papers published pertaining to the chemical analysis of coffee aroma following roasting and infusion and more then 400 volatile compounds have been detected. Some reports (BIGGERS *et al.*, 1969) have shown that the quality of the beverage is correlated with the profiles of gas chromatography. The study of green coffee has received less attention, although some reports on the composition of chemical constituents and of enzyme activities in green coffee correlate with differences in cup quality (AMORIM and SILVA, 1968; AMORIM *et al.*, 1975). The chemical and biochemical aspects of the seed show promise in future selection of coffee varieties in regard to flavor.

Soluble coffee (instant coffee), which first appeared early in the 19th century and became popular during World War II, is mostly of the dried or dehydrated type. It is processed either by the spray-drying method or lyophilization. The latter process allows for improved retention of flavor and aroma. Blends are prepared in order to obtain specific combinations in accordance with the desired quality and price of the product. Robusta coffee is used in blends because of its economy and apparent high soluble solid content. The primary consumers of instant coffee are the United States (110000 t), United Kingdom (12000 t), West Germany (9000 t), Japan (6000 t), and France (5000 t).

1.4 Recent Trends in Coffee Research

Among the important genetic traits limiting coffee production are those pertaining to resistance of fungal diseases, e.g. coffee rust and coffee berry disease. In addition, root-knot nematodes, leaf miner, and coffee borer susceptibility are of considerable economic importance. Other traits affecting productivity are photosynthetic efficiency, water utilization and tolerance to soil acidity and to aluminum. Genetic programs pertaining to beverage quality have been hindered

by the absence of correlations between biochemical parameters and cup quality. Such information will be invaluable to the coffee geneticist selecting cultivars of better beverage quality.

The coffee geneticist is faced with a unique problem because the major cultivated species, *C. arabica*, is the only self-pollinated tetraploid in the genus *Coffea*. Although some genetic variability is found among the *C. arabica* cultivars, the bulk of variability in the genus occurs among the approximately 70 diploid, outbred wild species.

Recent advances in culturing somatic cells of plants now make it possible to resolve this problem and apply the techniques of microbial genetics to higher plants. These new developments include embryo culture, cell cultivation, selective plating, fixation, diploidization and regeneration of haploid cells, as well as protoplast isolation, fusion, and the development of new interspecific plantlets in vitro. Furthermore, liquid suspension cultures of coffee cells have much potential in biochemical investigations relating to cup quality and aroma studies.

2. The Establishment of Tissue Cultures

2.1 Media and Cultivation Procedures

The first work on coffee tissue culture was done by STARITSKY (1970), who successfully cultured three species of *Coffea* on Linsmaier and Skoog inorganic salts (1965) supplemented with sucrose (30 g/l), thiamine HCl (1 mg/l), L-cysteine HCl (10 mg/l), meso-inositol (100 mg/l), kinetin (0.1 mg/l), 2,4-dichlorophenoxyacetic acid (2,4-D, 0.1 mg/l) or naphthalene acetic acid (NAA, 1 mg/l). SHARP *et al.* (1973) used the same salt medium, but increased some of the organic components to establish coffee tissue cultures: sucrose (40 g/l), thiamine (4 mg/l), NAA (2 mg/l). Five % coconut milk was sometimes added to the culture medium in addition to 10 mg/l of L-cysteine HCl to control oxidation of the phenolics. The initiation of callus growth is greatly facilitated in the absence of illumination at a temperature of 28° C. Under these conditions callus cells readily proliferate and in some instances undergo either shoot or root morphogenesis. Addition of 0.1 mg/l benzyladenine, 0.5 mg/l indole butyric acid (IBA), and 100 mg/l casein hydrolysate to the medium have a beneficial effect on the development of embryos and plantlets (STARITSKY, personal communication).

The subsequent transfer of cultures to an illuminated environment promotes the development of chloroplasts, but appears not to be absolutely necessary for the maintenance of growth or for the induction of morphogenesis. Temperature is a very critical factor in the growth of coffee callus cells. High temperatures of 28° C favor growth and development whereas lower temperatures near 20° C result in the cessation of growth and stimulate the oxidation of phenolics. Cyclic illumination and temperature is recommended for the induction of normal shoot and root morphogenesis whereby an illuminated period of 12 h at 28° C and a

dark period of 12 h at 23° C is provided. A regular transfer schedule of approximately 35 days is suggested for the maintenance of rapidly growing coffee clones. Upon the onset of cellular ageing, cells become brown, and proliferation ceases.

2.2 Choice of Plant Parts

Soft internodes of orthotrophic shoots were initially described as the most suitable plant material for development of coffee callus cultures (STARITSKY, 1970). Nevertheless, callus growth is also obtained by culturing anthers, internodes from either orthotropic or plagiotrophic shoots, petioles, leaves, perisperm, endosperm, and fruits. Cultures obtained from leaf explants attached to petioles result in large amounts of friable callus cells. A summary of current success in the culture of the above mentioned tissues is listed in Table 2.

Disinfection of coffee explants from greenhouse plants can be accomplished using 1% Na-hypochlorite solution for 15 min followed by a washing in a solution of L-cysteine HCl (10-100 mg/l). In the isolation of asceptic anther material, floral buds are treated with detergent for removal of excess wax from the external surface, and then immersed for 5 min in 1% hypochlorite solution followed by a wash in L-cysteine HCl (10-100 mg/l). Mercuric chloride (0.1%) has been used for the disinfection of some plant parts, but Na-hypochlorite or ethanol (95%) is preferable because mercuric chloride has a toxic effect on coffee tissues. Plant material harvested from the field requires a much more rigorous sterilization procedure than do greenhouse-grown plants.

2.3 Control of Growth and Development

Orthotrophic shoots of *C. arabica*, *C. canephora* and *C. liberica* were cultured by STARITSKY (1970). Embryo and plantlet formation were observed only in *C. canephora* tissues, whereas only fast-growing callus cells were obtained in the other two species. The globular embryos formed in *C. canephora* tissues were delimited to the periphery of the callus (Fig. 1 A and 1 B). Perhaps cell isolation is a prerequisite to the formation of these embryos and it appears that in cultures with multiple embryos, a stimulatory or inducer substance is synthesized, which promotes the induction of additional embryos (STARITSKY, 1970). The plantlets obtained in *C. canephora* were transferred to soil and after a period of adaptation they were indistinguishable from the plants from which explants were obtained (STARITSKY, personal communication). The development of plantlets in one species out of three clearly suggests the existence of different potentialities among different coffee species.

Various coffee explants of Mundo Novo and Bourbon Amarelo, two important *C. arabica* cultivars currently cultivated in Brazil were used by SHARP *et al.* (1973). The morphogenic potential of these cultures varied according to the origin of the cultured explant used for callus proliferation. Shoot and primary root development originated in explants suocultured onto an auxin-free culture medium. SHARP *et al.* (1973) observed massive adventitious root development in leaf callus grown in either the presence or absence of NAA. Peripheral brown clumps

Table 2. Current success in the culture of haploid, somatic, and triploid tissues of subsections and species of the genus *Coffea*

Type of tissue	Subsection	Species	Success
Haploid tissue	*Erythrocoffea*	*C. arabica* (2*n*=44)	SHARP *et al.* (1973). Anthers: proembryo development; isolated pollen grains: poor growth.
	Pachycoffea	*C. liberica* (2*n*=22)	SÖNDAHL, MONACO and SHARP (unpublished data). Anthers: rapid callus proliferation.
	Mozambicoffea	*C. racemosa* (2*n*=22)	SÖNDAHL, MONACO and SHARP (unpublished data). Anthers: poor growth.
		C. canephora (2*n*=44)	SÖNDAHL, MONACO and SHARP (unpublished data). Anthers: poor growth.
		C. canephora × *C. arabica* (2*n*=44)	SÖNDAHL, MONACO and SHARP (unpublished data). Anthers and pollen: poor callus growth.
Somatic tissue	*Erythrocoffea*	*C. canephora* (2*n*=22)	STARITSKY (1970). Orthotrophic shoot explant: embryo and plantlet development.
			COLONNA (1972). Embryo culture.
		C. arabica (2*n*=44)	STARITSKY (1970). Orthotrophic shoots: fast-growing callus.
			SHARP *et al.* (1973). Petioles: callus development; leaves: callus and adventitious root development: green fruits: brown callus clumps; orthotrophic shoots: primary root and shoot development; plagiotropic shoots: failed to grow or slow-growing callus.
			TOWNSLEY (1974). Stem explants: friable callus, abundant proliferation in cell suspension culture.
			MONACO *et al.* (1974). Perisperm explants: white friable callus.
			CROCOMO *et al.* (1975). Orthotrophic shoots: callus and shoot development; leaves: callus formation.
	Pachycoffea	*C. liberica* (2*n*=22)	STARITSKY (1970). Orthotrophic shoots: rapidly growing callus.
		C. dewevrei (2*n*=22)	COLONNA (1972). Embryo culture.
	Melanocoffea	*C. stenophylla* (2*n*=22)	MONACO *et al.* (1974). Perisperm explants: white friable callus.
Triploid tissue	*Erythrocoffea*	*C. arabica* (2*n*=44)	KELLER *et al.* (1972). Endosperm explants: callus formation.

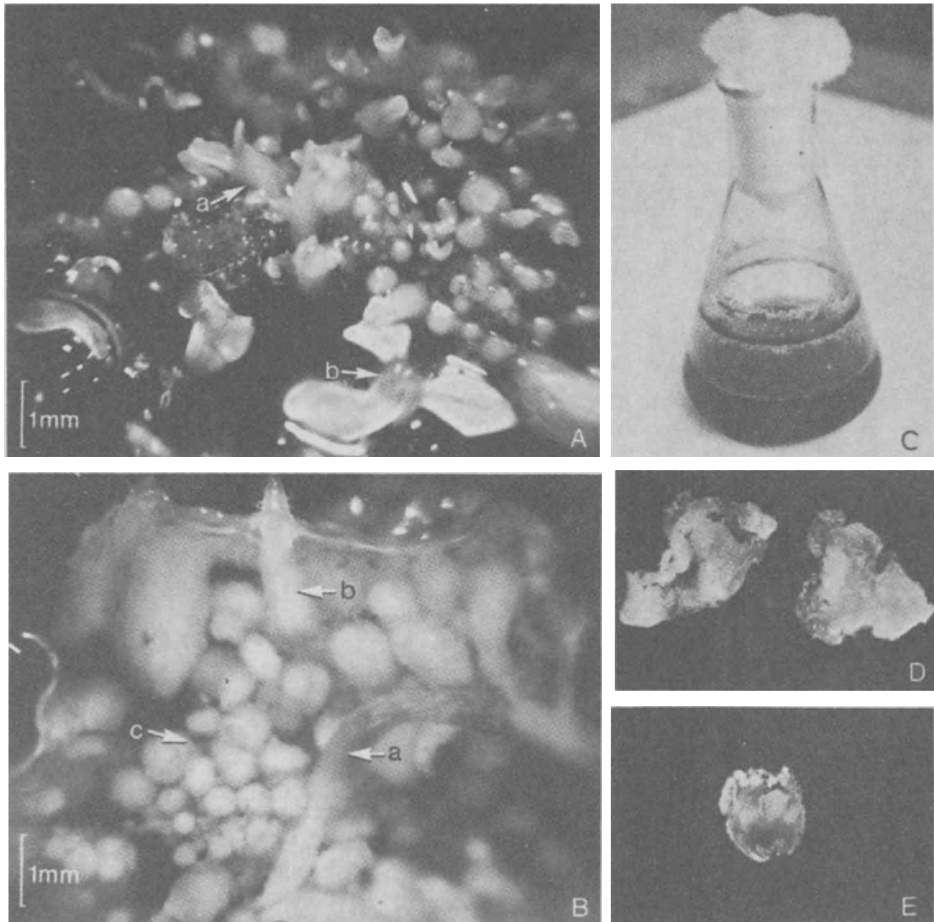

Fig. 1. (A) Embryo development in *Coffea canephora; a* embryo with two leaf primordia; *b* plantlet with three "cotyledons". (Photograph courtesy of STARITSKY, 1970a). (B) Embryo and plantlet development in *C. canephora*; *a* small plantlet with cotyledons, hypocotyl and primary root; *b* embryo with developing primary root; *c* globular embryo (Photograph courtesy of STARITSKY, 1970a). (C) *C. arabica* cells in suspension culture (Photograph courtesy of TOWNSLEY, 1974). (D) Callus culture obtained from 7-week-old cultured perisperm explants of *C. arabica* cv. Laurina; callus grown on 2,4-D free medium *(left)* and callus grown on 2,4-D supplemented medium *(right)*. (E) Development of perisperm and endosperm tissue on an 8-week-old explant from a developing fruit of *C. arabica* cv. Mundo Novo

of callus cells originated from explants of green fruits which failed to undergo subsequent morphogenesis.

More recently, CROCOMO *et al.* (1975) have reported the occurrence of five cellular phenotypic variations in callus cultures of *C. arabica* obtained from cultured explants of orthotrophic shoot and leaf explants in Staritsky's medium (1970) supplemented with hydrolyzed casein. Shoot development occurred in a few subcultured explants of orthotrophic shoots grown on the same medium plus White's vitamins (WHITE, 1963), 0.05 mg/l kinetin in the absence of 2,4-D.

Careful selection of suitable *Coffea* explants is required in experiments pertaining to the induction of shoot morphogenesis. The observation of limited shoot morphogenesis in cultures containing internodal explants derived from orthotrophic shoots suggests that such development originates from arrested shoot primordia. Characteristically, coffee plants have multiple arrested plagiotrophic and two orthotrophic shoot primordia at each node. The plagiotrophic primordia occur only after the 11th node in the developing seedling. Conversely, the occurrence of the two orthotrophic primordia can be observed beginning at the cotylendonary node. The removal of the apical meristem results in the development of the most apical orthotrophic primordium which would explain the development of shoots from internodal explants cultured on auxin-free medium. That is, unless the explants were obtained from the middle region of the internode.

2.4 Problem of Phenolic Oxidation

Coffea in addition to other tropical plants, contains a high concentration of phenols. These compounds always contain at least one hydroxy group on the benzene ring as exemplified in the case of the acids: chlorogenic, caffeic, ferulic, p-coumaric, protocatechuic, and transcinnamic. Several enzymes oxidize phenols to quinones, i.e. monophenol oxidase (tryosinase) and polyphenol oxidase (catechol oxidase). During the redox reaction, the hydroxyl group is oxidized leading to the formation of quinone and water. The brown color that frequently develops in callus cell cultures of *Coffea* is due to the formation of these quinones which are well known to be toxic to microorganisms and are inhibitory to plant cellular growth. Other inhibitory actions possibly result from bonding between phenols and proteins and subsequent oxidation to quinones, resulting in the loss of enzyme activities (LOOMIS and BATTAILE, 1966). When culturing these species of plants, special precautions are necessary for preventing the accumulation of toxic products resulting from phenolic oxidation.

The function of the phenols is not well understood. Some participate in the formation of lignin (transcinnamic, caffeic, and ferulic), others occur combined with sugars (transcinnamic, caffeic, and p-coumaric). Chlorogenic and protocatechuic acids correlate with disease resistance of certain plants (SALISBURY and ROSS, 1969). In *Coffea* chlorogenic acid occurs at high concentrations in seeds (2.7–10.3%) and in leaves (0.3–1.7%). The total chlorogenic acid content and the relative proportion of its three isomers (chlorogenic, isochlorogenic, and neochlorogenic) are species-variable (LOPES *et al.*, 1974).

The phenolics and the phenolic oxidases of intact coffee tissues are apparently situated in separate pools or compartments isolated from one another inside the cell. Following wounding, cutting, or senescence of tissues, these pools are integrated and the oxidation process is initiated. During the preparation of primary coffee tissue explants or the sectioning of established cultures for transfer, tissues should be immersed in an isotonic medium supplemented with an antioxidant (L-cysteine HCl, dithiothreitol or Cleland's reagent, glutathione, mercaptoethanol, ascorbic acid, or diethyldithiocarbamate) for curtailing the oxidation of the phenolics. In addition to the presence of the reducing agent, the liquid medium reduces the exposure to oxygen and the isotonic medium minimizes cellular damage. High

temperature inside the transfer chamber should also be avoided because it appears to enhance the phenolic oxidation reaction. Furthermore, culture media should be fortified with L-cysteine HCl (10-50 mg/l) according to the type of tissue being used, and a concentration of 50-100 mg/l of L-cysteine HCl is recommended for the isotonic liquid wash medium used during the excision of explants or during subculturing. Coconut milk (5-10%) can also be added to the liquid wash medium for this procedure, however this is not encouraged on account of its undefined constitution.

Maintenance of cultures in the dark is also helpful because illumination is stimulatory to the production of phenolics. Both low sugar, 1% sucrose, and 22.1 mg/l 2,4-D (DAVIES, 1972) in the culture medium are effective in decreasing the endogenous tissue phenolic concentrations. Addition of activated charcoal at optimal concentrations of 1% (grade G-60) or 2% (Merck, A. G., Darmstadt) in the culture medium has been reported by ANAGNOSTAKIS (1974) and BAJAJ *et al.* (1975) to apparently prevent accumulation of inhibitory substances in the culture medium and to dramatically increase the frequency of androgenesis and the time prior to plantlet emergence in cultured tobacco anthers. SÖNDAHL (unpublished) has observed that the addition of activated charcoal (0.01-2%—grade G-60) to the culture medium has no detectable effect upon the growth or development of cultured *Coffea* leaf explants.

3. Suspension Culture

3.1 Media and Cultivation Procedures

Individual coffee cells were obtained from friable callus cultures derived from stem explants cultured in 100 ml of modified medium (GAMBORG and EVELEIGH, 1968). Erlenmeyer flasks (250 ml) were placed on a reciprocal shaker at 60 cycles per min with a 1 in circular orbit and maintained in the dark at 28° C (TOWNSLEY, 1974). The purpose of this work was to develop the production of coffee aroma in suspension cultures. The liquid and solid media used were those developed by GAMBORG and EVELEIGH (1968), PRL-4 plus 10% coconut milk and the synthetic B-5 medium. The composition of these media are listed below:

	PRL-4 (mg/l)	B-5 (mg/l)
KNO_3	1000	2500
$NaH_2PO_4 \cdot H_2O$	90	150
$(NH_4)_2SO_4$	200	134
$MgSO_4 \cdot 7H_2O$	250	250
$CaCl_2 \cdot 2H_2O$	150	150
KI	0.75	0.75
Na_2HPO_4	30	—
Iron (sequestrene 330 Fe)	28	28
Final pH	6.2	5.5

Micronutrients (mg/l): $MnSO_4 \cdot H_2O$ (10), H_3BO_3 (3.0), Zn $SO_4 \cdot 7H_2O$ (3.0), $NaMoO_4 \cdot 2H_2O$ (0.25), $CuSO_4$ (0.25), $CoCl_2 \cdot 6H_2O$ (0.25). *Vitamins* (mg/l): nicotinic acid (1.0), thiamine (10.0), pyridoxine (1.0). *Growth factors:* 2,4-D (2.0 mg/l), sucrose (20 g/l), myo-inositol (100 mg/l) and for PRL-4 medium N-Z Amine type A (12.0 mg/l). All culture media were sterilized by autoclaving at 15 psi for 15 min.

Addition of casein hydrolysate (0.2%) to the synthetic B-5 medium was stimulatory to cell proliferation. The PRL-4 medium was more successful than B-5 medium for coffee suspension cultures which were maintained for more than three years on a 1-2 week subculture schedule (Fig. 1 C). Throughout this growth period several variations in culture growth rate, cell yield, color, coffee aroma and its intensity after roasting have been noted. Rapidly dividing cell cultures have a light cream color which became darker as the incubation period increased. It was observed that the majority of new cells in suspension culture were formed via cell elongation and subsequent cell division. Active cell expansion and division was similarly obtained when 2,4-D was replaced with indole-3-acetic acid (IAA), kinetin, or both plus casein hydrolysate. One striking aspect was that IAA induced the accumulation of oil droplets within the cells. The cells achieved a maximum growth rate within 10-20 days depending on the stage of growth (i.e. logarithmic, stationary, etc.) of the inoculum and on the composition of the medium. Aromatic properties and to some extent taste characteristics of roasted green coffee was obtained in coffee suspension cultures growing on PRL-4 liquid medium plus 10% of coconut milk.

The exponential phase of growth occurs between days 4-8 with maximum cell yield occurring at day 8. Although the pH was adjusted to 6.0 prior to autoclaving, sterilized medium had a pH of 5.0. The pH showed a decrease during the first two days (pH 4.5), it increased during the exponential phase of growth (pH 6.0) and then decreased again. A longer lag period following transfer was observed if the pH of the initial culture was adjusted to pH 4.0 following sterilization.

3.2 Biochemical Research

The fatty acid composition of coffee suspension cultures was compared with intact leaves, stems, and green coffee beans (Voort and Townsley, 1974). The media employed for the culture of coffee suspension cultures were PRL-4 and two modifications of Fox's medium (Motoyoshi and Oshima, 1968). The growth hormone, 2,4-D was added to modified Fox's medium at a concentration of 2 mg/l to supplement IAA and kinetin already present. The other modification was the presence of 2,4-D (2 mg/l) alone as the growth factor. All culture media containing 2,4-D produced rapidly dividing cell suspension cultures indicating that it is a major growth promoter for coffee cell suspensions. The pH was adjusted to 6.0 before autoclaving. The cultures grew at 28° C in light or dark and both conditions gave similar cell growth rate, cell yield, and fatty acid composition of the neutral lipids. The fatty acid composition of coffee suspension cultures were found to be very similar to that found in stem and leaves, but differed from the seeds by the presence of linoleic acid and the absence of arachidic acid. It is interesting to

note that linoleic acid is not generally found in seeds but is one of the most abundant fatty acids in leaves. The total oil content extractable by hexane on a dry weight basis in beans, leaves, stem and suspended cells on the 7 th day of culture was 12%, 3.0%, 1.9%, and 2.4%, respectively. On the 14 th day, coffee cells produced less oil content (1.3%) and contained significant amounts of C_{14} and short carbon chained fatty acids which were absent in younger cultures. The authors followed the conductivity (mmho/cm × 100) of the media which revealed a mirror image of the growth curve, and, therefore, could be used to determine stages in the growth phase of the cultures.

Recently, coffee cell cultures of *C. arabica* have been shown to contain the three sterols, β-sitosterol, stigmasterol and campesterol as the major components of the unsaponifiable fraction of the cell lipid. Two diterpenoid alcohols, cafestol and kawheol which are major components of coffee bean oil unsaponifiables have been shown to be present in the plant cell cultures. The presence of the two diterpenoid alcohols in coffee cell suspension cultures, would suggest that the coffee cell cultures maintain their ability to synthesize compounds unique to the parent coffee plant (F. VAN DE VOORT and P. M. TOWNSLEY, personal communication).

4. Endosperm and Perisperm Culture

4.1 Development and Cultivation Procedures

Coffee fruit is a drupe normally containing two seeds derived from a bilocular ovary. Mature fruits have a thick pericarp consisting of the exocarp, the mesocarp, and endocarp tissue. The latter constitutes the hard external layer covering each seed, the "parchment". Under this parchment there is a thin seed coat (ca. 70 µm thick), the "silver skin", which ontogenetically originates from the single ovule integument (DEDECCA, 1957, 1958). Following fertilization in coffee, the nucellus degenerates and the multiple layered integument rapidly develops leading to the formation of the perisperm tissue. As seed development progresses, the perisperm is completely absorbed by the endosperm, and becomes the silver skin of the mature seed.

With the purpose of studying caffeine synthesis in endosperm tissue, seeds of *C. arabica* c.v. Bourbon were placed into 25 × 110 mm test tubes containing 20 ml of MURASHIGE and SKOOG'S medium (1962) supplemented with malt extract (500 mg/l) and casein hydrolysate (200 mg/l) (KELLER, *et al.*, 1972). The seeds were surface sterilized in 96% alcohol and 3% H_2O_2 for 5 min. Cross sections of 3–4 mm thick or entire halves of seeds without the pericarp were used as explants. The explants were cultured at $27 \pm 1°$ C, 60–70% relative humidity, 12 h, photoperiod using fluorescent illumination (Philips T4 40 W/17). Sections less than 2 mm in thickness failed to grow or grew poorly and then succumbed.

Explants from developing fruits with immature seeds of *C. arabica* (Mundo Novo and Laurina cvs.) and *C. stenophylla*, having a rapidly proliferating perisperm and undeveloped endosperm, were excised and placed in 45 ml French

square culture bottles containing 10 ml of LINSMAIER and SKOOG'S (1965) agar salt solution supplemented with sucrose (30 g/l), thiamine (1.0 mg/l), cysteine (40 mg/l), meso-inositol (100 mg/l), kinetin (0.1 mg/l) in the presence or absence of 2,4-D (0.1 mg/l). Cultures were grown in the dark at 25–28° C and in all instances underwent rapid cell proliferation resulting in a massive amount of cellular growth at the surface of the explant (MONACO *et al.*, 1974).

4.2 Current Success and Application

Using cross sections of immature seed, KELLER *et al.* (1972) observed that the formation of new cells occurred both on the surfaces of endosperm slices and pericarp slices in instances where the pericarp had not been damaged during disinfection. Areas of white callus cell proliferation occurred on the surface of these explants which completely covered the entire surface after three weeks. Microscopic examination revealed that these proliferating tissues originated from the endosperm and had a loose structure of individual cells which were either round or spherical shaped.

Apart from the size of the inoculum, the age of the fruit proved to be a decisive factor. Only young fresh fruits weighing between 300–500 mg with soft endosperm were satisfactory for cultivation. Material from older fruits ceased to grow and became dessicated after some of time. Good growth of primary fruit cultures was obtained using the modified Murashige and Skoog medium. The success of raising subcultures of *C. canephora* tissues suggested that this species is easier to cultivate than *C. arabica*.

Analyses of caffeine content indicated that synthesis does occur in the growing endosperm tissue cultured in vitro. Endogenous caffeine increases by a factor of three after two weeks and by a factor of six after 4–5 weeks. Endogenous caffeine of the endosperm tissue is rapidly transported to the growth medium where it increases in concentration to approximately 95–98% following a 4–5 week culture period.

MONACO *et al.* (1974) have observed differences in the rate of cell proliferation and texture of cultured perisperm tissues. The latter varies from a white friable callus to a cream colored solid callus. The most striking observation was that coffee perisperm tissue proliferates rapidly in the absence of auxin, suggesting that this tissue is autonomous for auxin, and has sufficient endogenous auxin for sustaining growth (Fig. 1 D). As a practical application, perisperm culture can be utilized in many tissue culture studies as a source of fast-growing and friable callus. In some instances, the development of the young endosperm in the internal part of the perisperm was observed in *C. arabica* cv. Mundo Novo (Fig. 1 E). The endosperm which develops interior to the perisperm near the fruit base can be easily excised prior to the initiation of primary cultures and thus avoid the occurrence of mixed cellular populations.

All attempts to subculture endosperm isolated from growing perisperm cultures were unsuccessful and primary endosperm explants obtained from older seeds and placed into culture developed a brown coloration and succumbed.

Perhaps the supposed high endogenous auxin content of perisperm prevents the onset of phenolic oxidation when the perisperm and endosperm are cultured together.

5. Embryo Culture

5.1 Media and Cultivation Procedures

Embryo cultures of *C.canephora* cv. Robusta and *C.dewevrei* cv. Excelsa and cv. Neo-arnoldiana have been studied in solid and liquid Heller's salt solution (HELLER, 1953) plus sucrose (20 g/l), meso-inositol (200 mg/l), thiamine HCl (1.0 mg/l), cysteine HCl (2.0 mg/l). Calcium pantothenate (0.5 mg/l), nicotinic acid (1.0 mg/l), adenine (1.0 mg/l), pyrodoxine HCl (0.8 mg/l) and glycine (3.0 mg/l) (COLONNA *et al.*,1971; COLONNA, 1972). The cultures were maintained under a 12h photoperiod at 28° C day and 21° C night temperature. *C. arabica* (cv. Catuai) embryos were cultured in LINSMAIER and SKOOG's (1965) inorganic medium with the addition of sucrose (30 g/l), myo-inositol (100 mg/l), thiamine (1.0 mg/l), cysteine (40 mg/l), kinetin (0.1 mg/l) and 2,4-D (0.1 mg/l) or NAA (2.0 mg/l), agar (0.8%), pH 5.5 (SÖNDAHL, MONACO and SHARP, unpublished data).

5.2 Techniques and Embryo Development

Coffee seeds are surface sterilized and then soaked for 36–48 h in sterile water before the embryos are removed aseptically. If this procedure is followed, it is suggested to use 95% ethanol for 3 min with the application of a vacuum (1 min) and then soak in 0.7% hypochlorite for 5 min (COLONNA, 1972). Mercuric chloride (0.1%) for 15 min plus 5 min of vacuum was also used with success, but this treatment was too strong and resulted in an abnormal growth or arrested embryos. Alternatively, coffee seeds after removal of the parchment and silver skin can be soaked in tap water for 12 h, their embryos excised, and subsequently sterilized. In this case 1% hypochlorite for 6 min followed by three rinses in sterile water was successful.

These studies showed that *C.canephora* embryos had a slower rate of development than *C.dewevrei* embryos which presented maximum growth at the 60–70 th day of culture. Subculturing was necessary to provide for continued development. The liquid medium provided longer root growth, but the development was more regular in agar medium. The first pair of leaves in both varieties developed on the 45 th day of culture and on the 75 th day a second pair of leaves appeared in *C.dewevrei* (cv. Excelsa). The addition of 2×10^{-6}M IAA to the culture medium promoted a 17% increase in elongation, while 10% coconut milk in addition to the IAA produced a 38% increase in elongation at the 40 th day of culture.

In *C.arabica*, normal embryo development resulted with limited hypocotyl elongation. Callus formation at the intersect between the root and hypocotyl

region occurred in a few instances at later stages of embryo development (cotyledons fully opened). Root formation occurred most frequently in NAA containing medium (SÖNDAHL *et al.*, unpublished data).

5.3 Effect of Caffeine

The effect of caffeine was studied in immature embryos of *C. canephora* cv. Robusta and *C. dewevrei* cv. Excelsa and cv. Neo-arnoldiana cultured on COLONNA (1972) medium supplemented with caffeine concentrations of 0, 10^{-6}, 10^{-4}, and 10^{-2}M. The endogenous caffeine content of immature seeds from these three cultivars, on a dry weight basis was 2.8%, 1.0%, and 1.3% respectively. The elongation of cultured *C. dewevrei* embryos and Robusta embryos were inhibited by exogenous caffeine concentrations of 10^{-4} to 10^{-2}M and 10^{-6} to 10^{-2}M, respectively. However, growth on a fresh weight basis was inhibited by exogenous caffeine concentrations between 10^{-4} to 10^{-6}M for all three cultivars. A mild stimulatory effect of caffeine on the total length and gain in fresh weight of embryo cultures of *C. dewevrei* cultivars containing caffeine concentrations between 10^{-6} to 10^{-4}M was observed. Furthermore, it is interesting to note that this species has a lower caffeine content than *C. canephora* (approximately a two-fold reduction).

6. Haploids

6.1 Media and Cultivation Procedures

Floral buds 0.5–0.6 cm in length of *C. arabica* cv. Bourbon Amarelo containing anthers approximately 0.45 cm in length with predominantly uninucleate pollen grains were surface sterilized in 1% hypochlorite for 5 min, rinsed, and immersed in sterile solutions of L-cysteine HCl (10 mg/l) or sterile coconut milk (5%). The anthers were removed asceptically with the aid of a dissecting microscope and glass dissecting needles, and placed in culture. The submersion of coffee buds in either L-cysteine HCl or coconut milk solutions during all transfer operations and the use of glass needles served to minimize the oxidation of the phenolic compounds and thus curtailed the subsequent formation of toxic products. Anthers of *C. liberica* were prepared for culture using the same technique except that only liquid medium containing 100 mg/l of L-cysteine was used in the rinsing and dissecting solutions. All the measurements of anther length were made prior to transfer.

Individual microspores of both *C. arabica* and *C. liberica* were cultured using the nurse culture technique described by SHARP *et al.* (1972). Ten to twenty microspores were "nursed" on filter paper discs placed on top cultured intact anthers of the same species or upon fast-growing *C. arabica* cells.

Anthers of *C. arabica*, cv. Bourbon Amarelo, were successfully cultured on LINSMAIER and SKOOG's (1965) inorganic salts, sucrose (40 g/l), meso-inositol (100 mg/l), thiamine HCl (4.0 mg/l), L-cysteine HCl (10 mg/l), kinetin (0.1 mg/l), and 2,4-D (0.1 mg/l); agar (0.8 g/l) at a pH of 5.8 before autoclaving (SHARP *et al.*, 1973). Cultures were maintained in darkness at 25° C.

C. liberica anthers at three different stages of microsporogenesis were cultured on the above medium containing 3.5 mg/l of 2,4-D and 40 mg/l of L-cysteine. Recently, *C. arabica* anthers cv. Mundo Novo have been cultured on the same medium (SÖNDAHL, MONACO, and SHARP, unpublished data).

6.2 Current Progress

Rapidly growing, friable, white callus cells with the di-haploid chromosome complement (2X = 22) were obtained from cultured anthers of *C. arabica* cv. Bourbon Amarelo. Some cell lines of Bourbon Amarelo showed an uncommon chromosome reduction in vitro having the haploid complement (X = 11). Di-haploid callus cells from cultured anthers appeared to undergo abundant proembryo formation on auxin-depleted medium, however, such proembryos failed to undergo subsequent development. Individually cultured microspores did not grow well for either *C. liberica* or *C. arabica*. In the latter species only one nurse culture out of 100 showed cell proliferation.

A correlation between floral bud size, anther size, and stage of microsporogenesis occurs in *C. liberica* (Fig. 2). Anthers at the pollen mother cell stage, Leptotene I, and the binucleate stages of development were placed in culture and a correlation was found between the stage of microsporogenesis and the amount of callus growth following a 90-day growth period (Table 3). Comparative work using the microspore stage of anther development still needs to be studied.

The same medium was used to culture anthers of *C. arabica* (2X = 22), *Coffea racemosa* (X = 11), colchicine doubled *C. canephora* (2X = 22), and *C. canephora* × *C. arabica* (2X = 22). These cultures grew poorly or failed to grow which confirms that different coffee species and perhaps cultivars have specific requirements for growth in vitro (SÖNDAHL *et al.*, unpublished data).

Table 3. Relationship between bud length, anther length, stage of microsporogenesis, and average fresh weight of *C. liberica*

Bud length (cm)	Anther length (cm)	Number of replicate cultures	Microsporogenesis stage	Average fresh weight (g)
0.40	0.32	9	pre-meiosis	0.3857
0.87	0.65	17	Leptotene I	0.6780
1.85	0.92	18	binucleated	0.9331

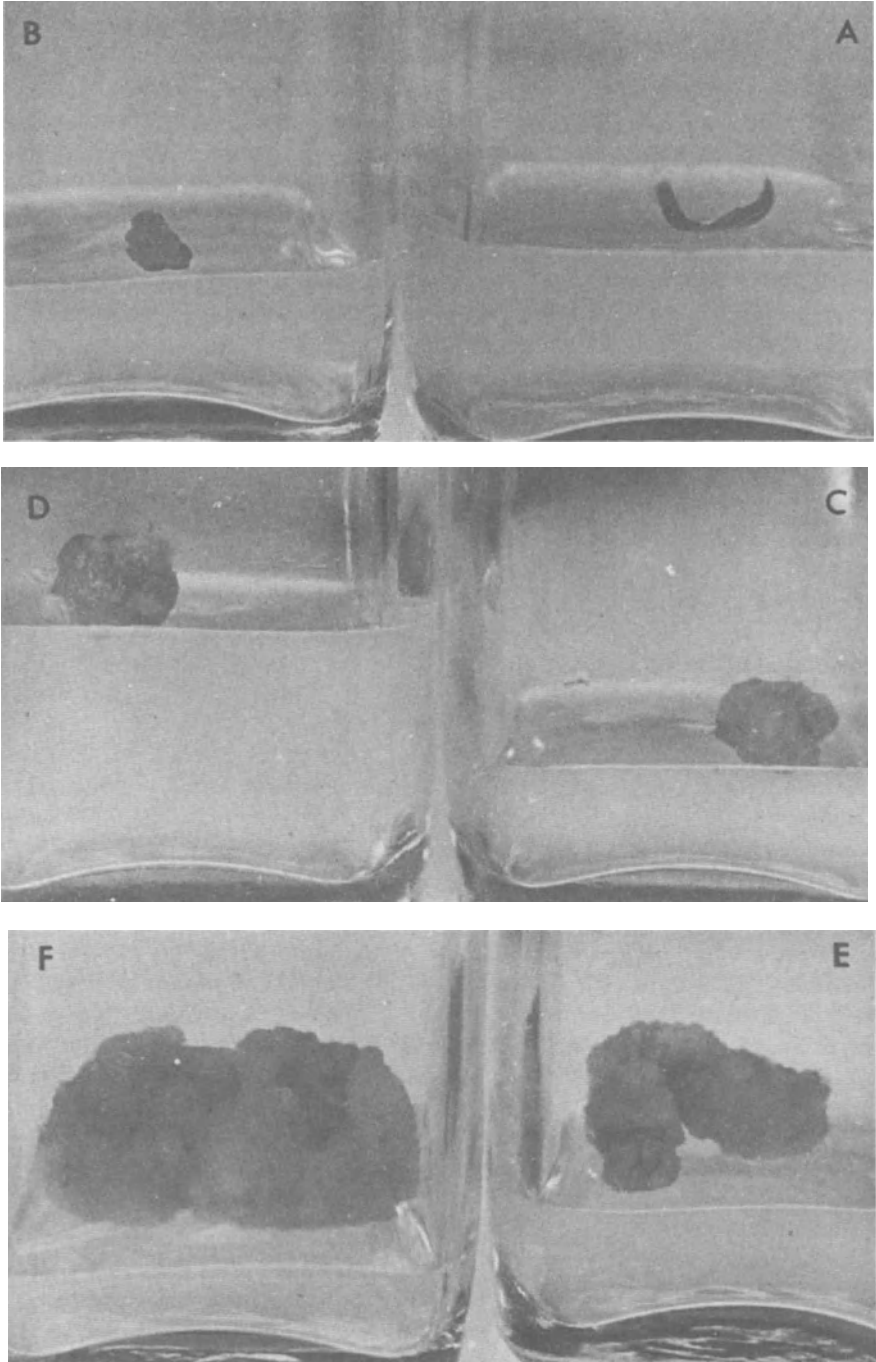

Fig. 2A–F. *C. liberica* callus from anther cultures following 3 months in culture: (A) and (B) anthers at the pollen mother cell stage; (C) and (D) anthers at the early meiosis (Leptotene I); (E) and (F) anthers cultured at the binucleate pollen stage

7. The Feasibility of the Tissue Culture Approach to Coffee Crop Improvement

Recent advances pertaining to the culture of somatic cells in higher plants now open the possibility of applying the techniques of microbial genetics for the development of improved plants. These new developments include the cultivation, regeneration, selective plating, and diploidization of haploid cells, as well as protoplast formation, fusion, and the uptake of exogenously supplied molecules and particles (see Chap. IV of this Vol.). Coffee geneticists are interested in the application of these techniques for the generation and selection of genetic variability with the goal of altering the genetic constitution in cultured coffee cells in an attempt to generate new cultivars.

Coffee genetics and breeding have been carried out in several countries, however, one of the most thorough programs for *C. arabica* has been conducted at the Institute o Agronômico de Campinas. Because of its perennial habit, progress in coffee breeding and coffee genetics has been hindered by the long time required for genetic analysis and the selection of new cultivars. Despite these restrictions, more than fifty mutants affecting various morphological characteristics, have already been isolated, and the mode of inheritance of these mutants has been studied. Some of the genes, like *Ct* (Caturra) and others for semi-dwarf growth, have been intensively used in these breeding programs. Other genes conditioning resistance to disease and pests namely rust, leaf miner, and root-knot nematodes are also of great interest.

Among the biochemical mutants, attention has been given mainly to obtain caffeine-free mutations which has resulted in the isolation of a reduced caffeine mutant in *C. arabica* cv. Laurina. The caffeine content in the normal arabic genotype is 1.2% but the gene *lr* (laurina) reduces caffeine to 0.6%. The existence of a caffeine-free species in the genus *Coffea* offers promise for the development of similar variation within other economic cultivars of *C. arabica.*

Breeding has been carried out for many years aiming at the selection of cultivars of *C. arabica* with a capacity for high yields. The success achieved can be evaluated by the development of the three new cultivars Mundo Novo, Catuai Amarelo, and Catuai Vermelho that yield 200% more than the original Arabica cultivar which is still cultivated in Latin America. At present, emphasis has been placed on the improvement of specific characteristics, e.g. genetic resistance to disease and pests, uniform ripening, suitability for mechanical harvest, tolerance to manganese and aluminum toxicity, tolerance to acid soils, high soluble solids content, and caffeine-free selection.

In Brazil there is increased interest in the cultivation of *C. canephora* which is cultivated commercially in the State of Espirito Santo. Because of its adaptation to areas where *C. arabica* cannot be grown economically, trials are being conducted to establish the potentialities of this species under various climatic conditions prevailing in the tropical areas of Brazil. The existing germplasm is very restricted and a special breeding program has been established to improve the genetic constitution of this cultivar for the coffee growers. Since *C. canephora* is an allogamous self-incompatible species, the breeding methods are different from the

ones used for *C. arabica* and emphasis is given to population breeding or hybrid production (CARVALHO and MONACO, 1969).

The induction of genetic variability and the accomplishment of gene transference, the basis for breeding in *C. arabica*, are difficult. In this species, 90% of the flowers are self-pollinated as determined by experimental work using the gene marker *cera* (CARVALHO and MONACO, 1962). In addition to that, the breeding programs for *Coffea* require long time allotments for the selection and reproduction of desirable cultivars. The use of cell and tissue culture techniques have potential in resolving these difficulties. Two aspects of this approach should be considered: (1) tissue culture vegetative reproduction for clonal maintenance and (2) manipulation of genetic variability.

The use of tissue culture techniques for the vegetative propagation of desirable genotypes in *C. arabica* is of little importance because of the pattern of self-pollination occurring in this species, which generates homozygosity. However, attempts are being made to develop hybrid coffee seeds. This of course is impractical until male sterility is established to allow for outbreeding and the subsequent increase of genetic variability among progeny. At this time, tissue culture technique for vegetative propagation of *C. arabica* will be of commercial importance in the propagation and cloning of important genotype combinations. Utilizing male sterility, hybrids containing various combinations of the six known dominant genes for rust resistance can be accomplished. The genotypes could then be perpetuated using the tissue culture vegetative propagation technique.

In *C. canephora*, the tissue culture technique for vegetative reproduction has particular application in this highly heterozygous, allogamous, self-incompatible species. This approach should be invaluable for selection and fixation of interesting genotypes.

Cultivation of microspores obtained from complex hybrids of *C. arabica* is of special interest in regard to the coffee rust disease because these hybrids resulted from the incorporation of resistance genes of wild coffee using artificial pollination. The objective of such crosses, of course, is to combine multiple desirable genes in a single genotype. For an example, six known dominant genes confer rust resistance. Here 1/64 of the microspores in F_1 from a hexahybrid mating will carry all such genes provided there are no linkages. Upon development of appropriate techniques for the selection of microspores with desirable genotypes, doubling of the chromosome complement, and regeneration, propagation of desirable genetic information will be readily obtained. Furthermore, the resulting totally homozygous diploids will, of course, have a double dose of the genes for resistance and will have advantages over their parent lines because of a larger spectrum of resistance and the phenomenon of gene dosage.

Moreover, haploid selection (Fig. 3) may be used as an initial step in the eventual achievement of plasticity in a population (see Chap. II.1. of this Vol.). This may be of great value in the species *C. canephora* where self-incompatibility is of the gametophytic type. The cultivation of microspores should allow for the selection of clones with different self-incompatibility alleles. The induction of chromosome duplication followed by plant regeneration would result in plants which are homozygous for these incompatible alleles. Subsequently these plants are crossed two by two which would result in obtaining self sterile F_1s.

	Alleles			
Haploids	S_1	S_2	S_3	S_4
Diploids	S_1S_1	S_2S_2	S_3S_3	S_4S_4
Diploid F_1 Hybrids	S_1S_2		S_3S_4	
Double Cross Hybrids	$S_1S_3 : S_1S_4 : S_2S_3 : S_2S_4$			

Fig. 3. The homozygous plants of the diploids are crossed two by two and all the seeds from a given cross will have the same genotype. The double cross hybrid will be obtained by crossing self-sterile F_1 s. The double hybrids are desirable because they contain more genetic variability in the population and possess better plasticity than the original population

Crossing of such F_1s would result in the development of double cross hybrids with increased genetic variation which would allow for better plasticity in the population. The plasticity in these genotypes is because of their heterozygosity (Bradshaw, 1965).

If a haploid line of a disease-resistant heterozygous wild species, having one or more genes for vertical rust resistance is used, the product following diploidization should have a higher potential in field utilization, if there is a gene dosage effect. Furthermore, it should be pointed out that microspore culture and haploid regenerated plants will be useful in genetic analysis for the study of the expression of individual genes.

An example of haploid culture and the use of mutagens combined is a situation whereby an induced mutation in either a haploid cell line of *C. canephora* or a di-haploid cell line from one of the highly productive *C. arabica* cultivars followed by selection and diploidization can result in the development of new cultivars.

Embryo culture is one of the important ways of dealing with genetic variability in *Coffea*. The cultivation of immature embryos resulting from interspecific crosses in the same subsection or between subsections in the genus *Coffea* has much potential for obtaining new hybrids. Studies performed in Campinas indicate that a low incidence of success occurs in crosses achieved between species of the subsection Erythrocoffea (Carvalho and Monaco, 1968). Currently, investigators at Campinas are interested in the transference of the caffeine-free characteristic of the species *C. bengalensis* (Section Paracoffea) to *C. arabica* (Section Eucoffea, Subsection Erythrocoffea). One approach to this is to develop *C. bengalensis* tetraploid seedlings for crosses with *C. arabica* and the cultivation in vitro of the supposed abortive embryos due to a sporophytic incompatibility. One could also cross *C. bengalensis* directly with di-haploid plants of *C. arabica* followed by chromosome number doubling, provided that *C. arabica* di-haploids are not self-incompatible.

Numerous communications exist discussing the use of protoplasts as a tool to increase genetic variability (see Chap. IV of this Vol.). This can be pursued by means of (1) cellular interspecific or intergeneric hybridization or (2) cellular transformation by the introduction of foreign genetic material.

An interspecific protoplast fusion whereby the desirable traits of the heterozygous outbred diploid coffee species can be introduced into *C. arabica* using so-

matic protoplasts and di-haploid protoplasts derived from pollen would be such a situation where somatic mesophyll protoplasts of *C. canephora* (2×) or another wild diploid species are fused with di-haploid pollen protoplasts of *C. arabica* (2×). This would result in obtaining interspecific fusion products (4×) containing genetic material from *C. canephora* or another wild species and *C. arabica.* That is, assuming completion of a successful fusion followed by appropriate selection procedures. Interspecific plantlets could be subsequently obtained using appropriate hormonal treatments.

References see page 207.

7. Large Scale Propagation of Strawberry Plants from Tissue Culture

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1. Introduction

1.1 Importance of Strawberry Plant Culture

During the last 50 years, the culture of strawberry plant (*Fragaria ananassa*) has considerably fluctuated in terms of predominance of one cultivar or another. Since 1965, the introduction of the cultivar (cv.) Gorella in Western Europe has caused a considerable increase in the fields under cultivation. At the same time, the development of strawberry plant culture in the Mediterranean region was enhanced by the introduction of Californian cultivars, notably the cv. Tioga (LEMAITRE, 1973) which accounts for more than 60% of the marketed culture.

The techniques of cultivation have progressed equally in recent years. They are oriented especially toward the production of commercial fruit, as necessary for quality-conscious consumers, and which have helped to cut down the harvest time substantially. It is this latter factor that often impedes the expansion of the culture of strawberry plants in the industrialized countries.

The limiting factors which increase yields, including a considerable percentage of large fruit, are according to LEMAITRE, 1974: (1) an annual culture, (2) plant of high quality, and (3) a carefully determined planting season.

Each year before August 15th, Belgium plants 100 million plants, to cover its 3000 hectares of strawberry plants. But, at this time of the year, plant growers do not dispose of sufficient quantities of fresh plants. The strawberry plant grower is therefore obliged to grow runners himself, and this threatens their health. Cold storaged plants uprooted in winter and stored in the refrigerator enable an easy supply at the most favorable planting period. As a result, this type of plant has considerably increased in recent years. It is most suited in dry regions where transplantation is very difficult. However, according to MOLOT *et al.* (1973), a cold-stored plant is highly sensitive to *Phytophthora cactorum.*

1.2 Production of Healthy Strawberry Plants

Although, grown for many centuries the strawberry plant can only be multiplied by vegetative method. This explains the great dissemination of parasites such as virus and mycoplasma diseases, nematodes, soil fungi and tarsonems which are transmitted directly or indirectly to the descendants.

The impossibility of efficiently fighting against such parasites in large fields, the introduction of non-resistant cultivars, of the international trade and the in-

creasingly new numerous planting techniques, have rendered the problem more and more acute. However, most of strawberry-producing countries have drawn up a program of certification which takes various parasites into account.

2. The Principal Parasites Transmitted by Plants

2.1 Viruses and Mycoplasmas

A recent article (AERTS, 1974a) recorded 54 viruses and 8 mycoplasmas on strawberry plants, most of which have been widely commented on (FRAZIER, 1970). These diseases are almost all epidemic, transmitted by aphids or nematodes, or even by cicads in the case of mycoplasmas. If strawberry plants are generally tolerant in the presence of a single virus, they often react very violently in the presence of complexes. AERTS (1974a) pointed out reductions of yield between 20 and 80% according to the cultivar concerned. The two most widespread viruses are "strawberry mottle" and "strawberry yellow edge". Most diseases formerly referred to as "degeneration" were probably caused by the presence of complexes of virus in the planting stock. So far, no resistant cultivar has been found.

2.2 Nematodes and Tarsonems

Foliar, stem and endoparasitic root nematodes, like tarsonems, are frequently found in strawberry, and cause substantial decline in yield. Fighting against such parasites is difficult because of the absence of effective non-toxic pesticides, the apparent recovery of infected plants, and also the absence of resistant cultivars.

2.3 Soil-Borne Fungi

Red core *(Phytophthora fragariae)* and wilt of strawberry caused by *P. cactorum* or *Verticillium* are widespread in Australia, Europe and USA where they cause considerable damage. Only a few cultivars resistant to "red core" and "verticillium wilt" are known (PLAKIDAS, 1964). The contamination of a culture may be due to the soil or plants, and such diseases are difficult to detect as the fungi are inborn. In a field 60% infected, JORDAN (1974) noted that only 4% of the plants presented the symptoms typical of *Verticillium*. Moreover, it is not uncommon to find areas of infection in fields which have never yielded strawberry plants, or which have never shown external manifestation of symptoms on the culture of earlier strawberry plants.

Recently, MOLOT and NOURISSEAU (1974) have demonstrated the relationships between the physiological state of the strawberry plant and especially its content of growth regulators and the rate of contamination.

3. Traditional Techniques of Curing Plants

3.1 Chemotherapy

At present no chemical is known that can bring the various parasites listed above under effective control. Indeed, systemic fungicides can mask the symptoms of strawberry wilt disease, tetracyclines including those of mycoplasma diseases (HUBTANEN and CONVERSE, 1971), but, none of them can save the infected plants. Nevertheless, pesticides are very useful in preventive efforts. A pulverization scheme will help to avoid contamination in the multiplication fields grown in isolated areas.

Soil fumigation will prevent further contamination by soil parasites such as nematodes and fungi. These parasites can be completely destroyed with a mixture of chloropicrin and methylbromide (2/1) as recommended by WILHELM *et al.* (1961). Yet fumigation remains a dangerous process and some treatments can favor the spread of a disease, e.g. "*P. cactorum*" (MOLOT *et al.*, 1973).

3.2 Heat Therapy

Heat therapy has been practiced to eliminate strawberry viruses for over 30 years (POSNETTE and CROPLEY, 1958). Dipping plants in hot water (10 min at 46° C) is also a long-standing method of eliminating tarsonems and nematodes (STANILAND, 1953). More recently, GILLES (1971) developed an easy and efficient method for destroying tarsonems in large bulks of runners: dipping plants into a tepid solution (35–40° C) of kelthane (0.25–0.30%) for 10 min. Plant treatments with hot air or hot water cannot eliminate *P. cactorum* (MOLOT *et al.*, 1972).

As for viruses, strawberry plants are subjected to 37–38° C temperature for 1–4 weeks. Several techniques do exist and they can be related or not to developing dormant axillary buds (MCGREW and SCOTT, 1964; POSNETTE and JHA, 1960). The results of treatment depend on the treated cultivar, the healthy state of plants and the viruses. Some strains of crinkle virus and yellow edge virus are difficult to cure by heat therapy. Moreover, viruses can frequently appear several months after the treatment, or even 2.5 years later, on the apparently healthy plants (COMAN *et al.*, unpublished results; POSNETTE and CROPLEY, 1958; HILTON *et al.*, 1970).

To summarize, let us point out two particular methods which treat strawberry plants infected with viruses by using temperatures over 37–38° C. The treatment recommended by BOLTON (1967) helps to acclimatize plants to 50° C. Whereas in the method of JOHNSON and EGLITIS (1969), the inner tissues are heated by irradiation in a high-frequency field. GHENA *et al.* (1973) also used this method successfully to eliminate thermostable strains.

3.3 Conservative Selection

After heat therapy or meristem culture, healthy plants are isolated in screened cages, far from any source of contamination. The soil is disinfected by fumigation. Every mother plant is indexed, and daughter plants are then planted in the open

air, in well-isolated and disinfected plots. They remain under a very strict phytosanitary observation. The plants produced in this way constitute registered stock which is considered virus-free, though it is impossible to give absolute evidence (McGrew, 1959). This traditional method of multiplying healthy plants requires a minimum delay of 3 years before a healthy cultivar is at the producer's disposal.

4. Strawberry Plant Tissue Culture

4.1 Meristem Culture

In vitro culture of meristem has been the object of many attempts considering its practical advantage from the sanitary point of view. Belkengren and Miller (1962) were the first to recommend the elimination of viruses from strawberry plant by the meristem culture of *Fragaria vesca*. The same authors, one year later (Miller and Belkengren, 1963) published the results of their research on commercial strawberry plants.

If these studies are concerned with true meristem culture, it is also possible to regenerate the infected plants by means of small tip cuttings. Posnette and Jha (1960) eliminated resistant viruses by cutting the small axillary buds formed upon crown disks sliced from heat-treated plants. Miller and Belkengren (1963) eliminated a complex of viruses by excising 2–5 mm-long runner tips placed on culture medium. According to McGrew (1965), the elimination of strawberry latent C viruses from excised stolon-tips of different lengths depends on the duration of the heat treatment, but did not succeed in eliminating crinkle virus.

Other authors condition the success of curing strawberry plants by meristem culture to the previous heat therapy (Belkengren and Miller, 1962; Hilton *et al.*, 1970; Mullin *et al.*, 1974); however, this method has not been confirmed by other researchers. Adams and Stickels (unpublished results) grew a total of 55 meristem clones from plants infected with virus 4 (24 clones), virus 2 (25 clones), virus 1 (4 clones), viruses 1+3 (2 clones) and report that despite the absence of heat therapy all 55 meristem clones were freed from virus. Our research work gives the same conclusions: 47 meristem clones from 15 infected cultivars were freed without heat therapy.

Though previous heat treatment is not essential to eliminate virus diseases, however Vine (1968) and Mullin *et al.* (1974) mention a fast growth of meristem excised from heat treated plants. They also note better success in this case: heat treated 22%, untreated 5% (Mullin *et al.*, 1974); heat treated 50%, untreated 28% (Vine, 1968).

The size of explants explains some negative results for eliminating virus disease (Table 1). Molot and his coworkers (1972, 1973) eliminated *P. cactorum* by meristem culture provided that the size of explants was less than 0.5 mm.

The elimination of virus by meristem culture is definitive. Not a single case of virus resurgence has been reported. Belkengren and Miller (1962) and Boxus (unpublished results) have not found any virus 2.5 years after treatment; Mullin *et al.* (1974) 7 years after treatment.

Table 1. Factors influencing virus elimination

Size of the explant (mm)	Heat treatment	Virus elimination		Reference
?	none	55/55[a]	100%	ADAMS and STICKELS (unpublished)
0.2–0.4	none	47/47	100%	BOXUS (unpublished)
0.5	none	–	0%	HILTON *et al.* (1970)
0.5	6 weeks	–	77%	HILTON *et al.* (1970)
0.7–1.2	6 weeks	–	50%	HILTON *et al.* (1970)
0.5–1	none	7/19	37%	MILLER and BELKENGREN (1963)
0.5–1	38° C/120 h	1/ 3	33%	MILLER and BELKENGREN (1963)
3 –5	none	3/15	20%	MILLER and BELKENGREN (1963)
3 –5	38° C/19 h	1/ 1	100%	MILLER and BELKENGREN (1963)
0.3–0.8	36° C/6 weeks	28/34	82%	MULLIN *et al.* (1974)
0.3–0.8	none	2/ 8	25%	MULLIN *et al.* (1974)
0.2–0.3	none	–	100%	NISHI and OOSAWA (1974)
1	none	–	± 50%	NISHI and OOSAWA (1974)
< 0.8	with or without	–	± 100%	VINE (1968)

[a] Numerator = number of virus-free plantlets. Denominator = number of tested plantlets.

The use of very small explants requires adequate culture media. VAN HOOF (1974) mentions the importance of mineral equilibrium in the medium: 82% success on his medium, 10% on that of WHITE (1963), 31.5% on that of VINE (medium "A", first method), and 78% on the medium "B" of VINE (first method) (see Table 2).

Our results reveal the importance of growth substances (Tables 3a and b). The presence of benzylaminopurine (BAP) and an auxin, β-indolbutyric acid (IBA), is very stimulative, however, differences exist between cultivars. Among the 74 cultivars tested on the whole and representing 794 meristems excised in 1972–74, we obtained 462 rooted plantlets, that is 58% average (from 12 to 100% according to cultivar). In the only case of cultivar Hummi Grande, we did not obtain any plant (0/15) in 1973; but one year later, the same cultivar yielded 87% (7/8).

The negative results or failures of different researchers on the media by BELKENGREN and MILLER (1962) (10%), VINE (1968) (30%), NISHI and OOSAWA (1973) (8–13%), MULLIN *et al.* (1974) (5–22%), are mainly explained by the absence of BAP in the medium, whereas ADAMS (1972a) obtained 72% (26/37) in the presence of 0.1 mg/l BAP and MOLOT *et al.* (1973) 10–50% on our medium (0.1 mg/l of BAP). Coconut milk used by BELKENGREN and MILLER (1962) or VINE (1968) produced no better results.

The temperature of the growth chamber also influences results. ADAMS (1972a, b) succeeded at 25° C, but not at 19° C. We get better results at 28° C. The light required is not important. Daylight can vary from 12 to 16 h. A light intensity of 1000–1500 lux is sufficient.

After a 4-week period of culture, all the workers, except ADAMS (1972a) recommend a transplantation on a different medium for rooting. In our work, the second medium is not absolutely necessary, but its use increases the chances of

Table 2. Composition of meristem culture media

	ADAMS (1972)	BOXUS (1974)			MILLER and BELKENGREN (1963)	MULLIN *et al.* (1974)	
		Medium for meristems	Medium for budding	Basic medium for rooting		Medium A	Medium B
Macronutrients	MS[a]	Knop	Knop	Knop	White	Knop	Knop
Micronutrients	MS	MS	MS	MS	White	Berthelot	Berthelot
Vitamin mixture (mg/l)	WS	MS	MS	MS	White	Thiamine-HCl 1	See[b]
Coconut milk (%)	—	—	—	—	10	—	—
Hormonal substances	BAP 1	BAP 0.1	BAP 1	—	—	—	Kin 0.1
(mg/l)	IBA 1	IBA 1	IBA 1	IBA 1	—	IAA 1	IAA 2.5
	—	Gib 0.1	Gib 0.1	—	—	—	—
Sugar (g/l)	?	Gluc 40	Gluc 40	Gluc 40	Sacch 20	Gluc 30	Gluc 30
Agar (g/l)	0	8	8	8	7	0	0
pH	5.2	5.6—5.8	5.6—5.8	5.6—5.8	?	5.7—5.8	5.7—5.8

	NISHI and OOSAWA (1973)	VAN HOOF (1974)		VINE (1968)			
		Medium M	Medium T	I Med. A	I Med. B	II Med. A	II Med. B
Macronutrients	MS	see[c]	see[c]	MS	Knop	White	White
Micronutrients	MS	Heller	Heller	MS	MS	White	White
Vitamin mixture (mg/l)	LS	WS	see[d]	MS	meso-inositol 100	White	White
Coconut milk (%)	—	—	—	10	—	10	—
Hormonal substances	—	BAP 0.2	BAP 0.02	—	—	—	—
(mg/l)	—	IBA 1	IBA 1	IBA 1	—	—	—
	—	Gib 1	Gib 0.1	—	—	—	—
Sugar (g/l)	Sacch 30	Gluc 20	Gluc 20	Sacch 20	Gluc 40	Sacch 20	Sacch 20
Agar (g/l)	7	8	8	0	0	0	0
pH	6	?	?	5.6—5.8	5.6—5.8	5.6—5.8	5.6—5.8

[a] abbreviations: BAP = benzyladenine; Gib = Gibberellin; Gluc = Glucose; IBA = indolbutyric acid; IAA = indolacetic acid; Kin = kinetin; LS = Linsmaier and Skoog; MS = Murashige and Skoog; Sacch = saccharose; WS = Wetmore and Sorokin.

[b] id. MS mixture, but Thiamine 1 mg/l.

[c] (mg/l) = $MgSO_4 7H_2O$: 150; Na_2SO_4 : 450; KCl : 305; $(NH_4)_2SO_4$: 79; $Ca(NO_3)_2$: 560; KNO_3 : 373; NaH_2PO_4 : 500; $NaNO_3$: 240; NH_4NO_3 : 165; $CaCl_2$: 128; NaFe EDTA = 20.

[d] (mg/l) : nicotinic acid : 1; pyridoxine : 1; meso-inositol : 100; adenine : 5; biotine : 0.01; calcium pantothenate : 1.

Table 3a. Effect of benzyladenine on strawberry meristem culture

Cultivar	Basic medium	Basic medium + BAP (0.1 mg/l)
Bemanil	1/5[a]	1/3
Domanil	3/16	11/18
Fanil	1/20	20/25
Gorella	2/42	5/25
Goupil	2/28	5/14
Senga gigana	7/10	4/10
Surprise des Halles	10/18	13/13
Total	26/139 (18%)	59/108 (54%)

[a] Numerator = number of rooted plantlets, Denominator = number of excised meristems

Table 3b. Effect of benzyladenine and indolbutyric acid on strawberry meristem culture

Cultivar	Basic medium with BAP = 0.1 mg/l IBA = 1 mg/l	Basic medium with BAP = 0.1 mg/l IBA = 0.1 mg/l	Basic medium with BAP = 0.01 mg/l IBA = 1 mg/l	Total
Fanil	4/5[a]	4/5	2/5	10/15 (66%)
Domanil	4/5	3/5	1/5	8/15 (53%)
Gorella	2/5	0/4	0/4	2/13 (15%)
Surprise des Halles	5/5	3/5	4/4	12/14 (85%)
Total	15/20 (75%)	10/19 (52%)	7/18 (38%)	

[a] Numerator = number of rooted plantlets, Denominator = number of excised meristems

success. Frequently, keeping the explants on basal medium which contains cytokinin causes chlorosis of plantlets and can result in their death. The plantlets transplanted on the basal medium without any cytokinin or gibberellin turn green again in a few days and get roots within 3–4 weeks. Two to three months after the excision, the young plants can be planted into the ground (see Sect. 5.4).

The choice of meristems on the mother plant does not influence their further development. We have not observed any difference between meristems excised from lateral or apical buds of mother plant or from stolon as reported by VAN HOOF (1974). However, the excision is easier from young runner-tips than from well-developed plants. When the buds are excised from plants during their winter rest or from cold storage plants, a high percentage of meristems is internally infected. The traditional techniques of disinfection—15 min in a 5% solution of calcium hypochlorite after a brief dipping in pure alcohol for instance—are not effective to eliminate these internal infections.

4.2 Callus Culture

When NISHI and OOSAWA (1973) added an auxin (10^{-5} or 10^{-6}M of IAA, NAA or 2.4-D) or BAP (10^{-5}M) to their basal medium, meristems grew 2 or 3 weeks later and gave calli which could be sub-cultured and buds appeared on these calli 10–12 weeks later. This generation was related to the presence of BAP in the culture medium. Auxins inhibited this regeneration in part or completely in the case of 2,4-D. Callus has also been obtained by anther culture as described below.

4.3 Anther Culture

FOWLER *et al.* (1971) obtained a high percentage of calli from anthers excised from flower buds with petals completely concealed in the calyx. These calli originated from sporophytic tissues, and grew better in darkness than under light on the medium of NITSCH and NITSCH (1969) enriched with IAA (1 mg/l) and kinetin (1 mg/l). However, these calli did not regenerate any buds.

The Japanese workers (NISHI *et al.*, 1974; OOSAWA *et al.*, 1974) obtained anther callus cultures on different mineral media enriched with cytokinin (kinetin and BAP). The cultures were incubated under light 12 h a day. All the cultivars did not react to callus formation in the same way (12–70%). Root formation on callus sub-cultures was promoted by the presence of kinetin (10^{-5}M) and NAA (10^{-5}M) in the culture medium. Shoot formation appeared especially in the presence of BAP (10^{-5}M) and NAA (10^{-5}M) on LINSMAIER and SKOOG's medium (1965).

Differences exist between cultivars, but on the contrary no difference was observed according to the rate of virus infection. All the plants obtained from these cultures were identical with their parents, but they were virus-free. Mottle virus, mild yellow edge virus and crinkle virus were eliminated from 15 infected plants by anther culture. Also, these workers found that this process was more suitable than the traditional method of meristem culture: only one mother plant could regenerate a great number of virus-free plants.

ROSATI *et al.* (1975) have also obtained callus cultures from anthers excised at the first pollen mitosis stage. Medium number 1 of GRESSHOFF and DOY (1972) and the medium of LINSMAIER and SKOOG (1965), with 0.2 mg/l kinetin, 2.0 mg/l IAA and 0.4 mg/l, 2,4-D produce much more calli. Calli can be obtained under light. The regeneration of callus is relatively low and appears only on young cultures. Perhaps, the absence of BAP explains these negative results. The chilling treatment applied to the flowers before the excision of anthers highly increases the growth of callus and its potential for regeneration. All the plants are diploid, however, Italian workers believe that some of them have a gametic origin.

4.4 Fruit Tissue Culture

BAJAJ and COLLINS (1968) obtained in vitro formation of fruits by cultivating pollinated flowers on the complete medium of WHITE (1963). In the presence of giberellic acid (5–20 mg/l) unpollinated flowers also formed fruits, while NAA and
gibberellic acid (5–20 mg/l) unpollinated flowers also formed fruits, while NAA and

IBA did not induce parthenocarpic fruit. When kinetin and auxin were added to the basal medium, calli could be induced on the basal portion of the receptacle and flower pedicels or on tissue disks taken from the receptacle.

4.5 Micropropagation

ADAMS (1972b) stated: "it would be seen possible to obtain an unlimited number of plantlets from a single meristem." But he concluded: "Although the use of such methods could provide an extra insurance against infection by nematodes, viruses and other diseases, the current system of raising nuclear stocks in glasshouses has worked effectively for many years and is unlikely to be replaced."

Since then, NISHI and OOSAWA (1973), and BOXUS (1974) have reported mass production of virus-free strawberry plants by in vitro culture methods. Japanese workers proposed two different techniques, both of which refer to the regeneration of plantlets in callus cultures. On the medium of LINSMAIER and SKOOG + BAP 10^{-5}M, 80% of calli formed from meristems regenerate up to 50 plantlets (NISHI and OOSAWA, 1973), whereas a few of the anther calli can regenerate as much as 100 plantlets (OOSAWA *et al.*, 1974). After separation up to 86% of the newly formed shoots take roots on LINSMAIER and SKOOG'S medium without any cytokinin. The technique perfected by us (BOXUS, 1974) does not require callus culture. The buds are developed from lateral buds formed on the basis of each leaflet under the action of cytokinin (BAP, 1 mg/l). The number of buds formed in such a way is unlimited. If they are separated, they can make buds in the presence of BAP, or form roots in the absence of BAP. Rooting is 100% successful. The technique described in the following pages is applied to all 74 cultivars tested up to now.

The techniques of NISHI and OOSAWA (1973) present some disadvantages that we have overcome. Indeed, cells yield mutants more easily in callus cultures than in organized tissues. The presence of mutants is always to be feared for new formations. Moreover, the use of anthers as basal material increases the chances of "non corresponding copies" among material multiplied. Finally, these techniques require new and frequent excisions of meristems or anthers.

5. Industrial Multiplication of Strawberry Plant by in vitro Methods

The traditional production of strawberry plant faces many difficulties, some of which have already been reviewed. To overcome these, we have tried to use tissue cultures for the large scale production of strawberry plants. The technique we propose can be used industrially as practiced for orchids and for other plants (see Chap. I.2, 3 of this Vol.).

5.1 Description of Multiplication Principle

Multiplication is performed from strawberry plantlets isolated from meristems, stored on basal medium (see Table 2) and virus-tested. These plants are rejuven-

Fig. 1. 0.5 l jar containing more than 400 Cambridge Favourite buds, on basic medium enriched with 1 mg/l BAP

ated by suppressing all the old leaves and roots. They are then transferred aseptically to a fresh basal medium enriched with 1 mg/l of BAP.

Three to four weeks later, 2 or 3 axillary buds appear on the lower part of the petiole of the oldest leaves. These axillary buds grow very quickly and also produce new axillary buds within a few days. Within 6–8 weeks, the initial plantlet is transformed into a mass of more or less developed buds. Externally, these appear as a tuft of very small unifoliate leaves, with short petioles, closely pressed against each other, and at the lower part of these petioles, new buds are formed. There are no roots and no calli. These tufts can reach several cm in diameter, including 15 to 25 buds each of which has 2 or 3 leaves (Fig. 1).

These buds can be divided and separately transplanted onto a fresh medium. If they are planted in a medium containing cytokinin, the axillary buds continue to proliferate, but if they are planted on the basal one without cytokinin, the development of the axillary buds ceases immediately, and these buds produce normal young plants with roots and trifoliate leaves within 4–6 weeks (Fig. 2). This can be easily controlled by the addition or omission of BAP. Several cultivars have undergone this cycle (with or without cytokinin) for the last 3–4 years without showing any change in the type of growth. Up to now, all the 74 tested cultivars of *F. ananassa* have reacted in the same way. Recent tests show similar reactions with five clones of *F. vesca*.

By this process, it is possible to obtain several million plants from a single mother plant within one year (Fig. 3).

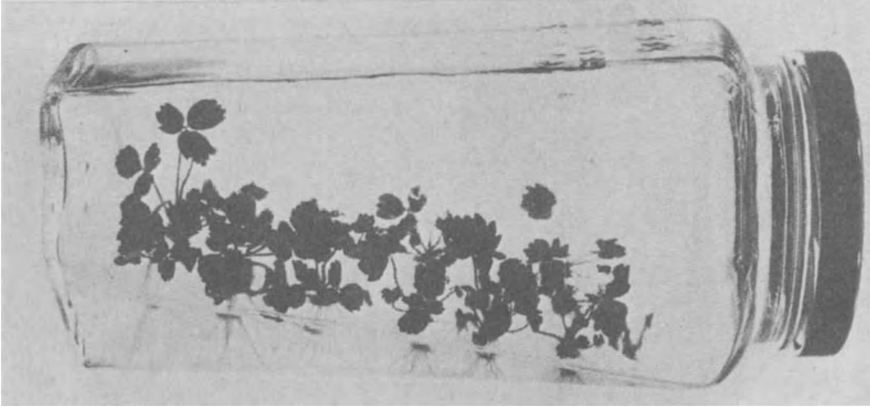

Fig. 2. Rooted plantlets of cv. Domanil, 5 weeks after transfer on basic medium

Fig. 3. Partial view of the growth cabinet for strawberry plants

5.2 Material and Methods

Our cultures were obtained with the same success in different types of glassware. We use a jar of 0.5 or 0.75 l with a 10-cm diameter (Fig. 1). This jar can contain 400 budding plantlets or 50–100 rooted plantlets.

The culture medium has previously been described (BOXUS, 1974; Table 2). It is solidified with 0.8% agar and adjusted to pH 5.6. The jars are autoclaved at 110° C for 15 min. Transplantation is carried out in a horizontal laminar flow bench. The temperature in the culture chamber is maintained at 24° C. Light is provided by fluorescent tubes of 40 watts 16 h in the daytime. Light does not seem to exert a great influence as the plantlets grow well, even under a weak light intensity, and show the same response under an intensity of 1000 or 5000 lux (provided by Osram, cold white tubes).

5.3 Conservation of Mother Plants

Mother plants of various cultivars are maintained in vitro, either in 150 × 25 mm test tubes, or in jam jars containing 15 ml and 60 ml of basal medium respectively. On this medium, young plants from meristems or regenerated by bud proliferation at first grow very fast forming some leaves and roots. Then, their growth slows down and stops after 2–3 months of culture. Plants can remain at this stage from several months to several years, without any care. They do not decay and there is no danger of contamination. It is then possible to transfer them on to a fresh medium after having suppressed the old leaves and roots. We have obtained excellent regenerations from plants which had been stored for two years on the same medium.

5.4 Transference of Plants to the Field

Adaptation of young plants to normal conditions of culture is very easy. Plants are transplanted on a leaf mould disinfected some time previously either in the open air or in greenhouses. They are maintained for a few days under a plastic cover or under water mist in order to avoid their dessication (Fig. 4). Transplantation will be 100% successful if the soil has not been disinfected recently. It is also

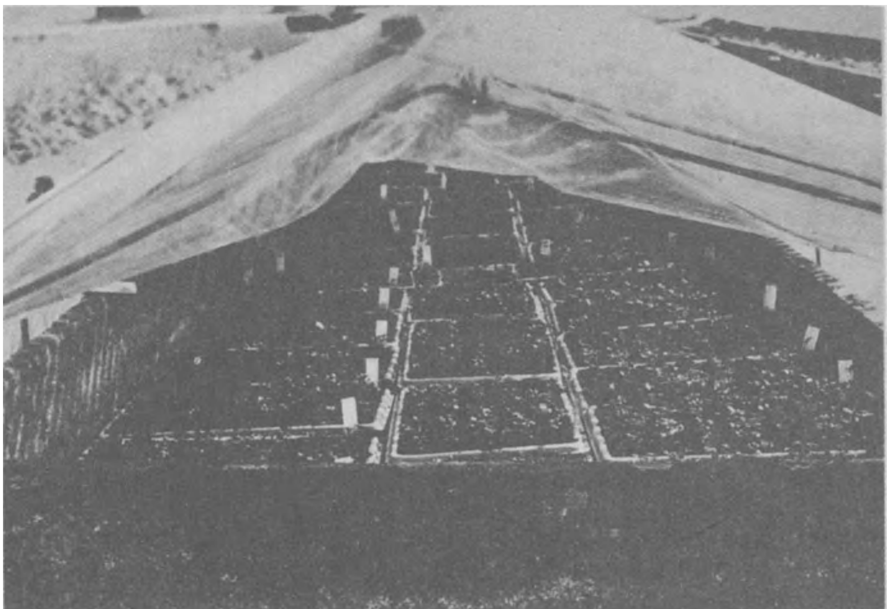

Fig. 4. Strawberry plants under plastic cover, 3 weeks after transplantation in soil (40 plants per tray)

possible to maintain young plantlets from jars for several weeks at the laboratory temperature or in a cold chamber before transplanting them. They are simply put in plastic bags and kept in darkness or in the light till the time of transplantation. One month after transplantation, plants can be transferred to the field. They have a healthy growth and a strong tendency to produce large numbers of runners.

5.5 Productivity Test

In the 1972–73 season, 400 young strawberry plants cv. Domanil, obtained by the above mentioned method were studied (by M. LINDEN—first breeder of this cultivar) and no mutations were observed.

During the next season (1973–74), we tested the productivity of 5600 plants, 4640 of which were obtained by micropropagation and 960 control plants. Those plants belonged to different cultivars. Each of them was represented by 1–6 "meri-clone" (clone of plants obtained from one meristem by micropropagation) plus one control clone.

Forty plants of each clone were grown in four plots on an experimental patch of 13 aeres. Statistical analysis of harvests did not reveal any significant difference between the clones of the same cultivar. However, this analysis showed significant differences between the plots of the same clone, differences being related to the heterogeneity of experimental field.

In the same test, the number of plants contaminated by *Verticillium* and *P. cactorum* did not reveal any particular sensitivity of meristem plants to these parasites.

5.6 Advantages and Drawbacks of this Method

Although it does not increase the production of plants as reported by BRINGHURST and VOTH (1972), the technique of in vitro micropropagation is profitable. It completely ensures the health of the plants, i.e. against virus diseases, nematodes, tarsonems and principally soil-fungi. The required phytosanitary treatments by traditional method does not offer such a security.

Moreover, in vitro micropropagation allows easy production planning. Millions of plants can be produced within a year from a few mother plants. The latter are kept by tens on a surface of 2 or 3 m^2, very cheaply, ready for use in all seasons, and always perfectly healthy.

Preservation and expedition of young in vitro plantlets in plastic bags opens new perspectives. The cost of their expedition will be negligible. The strawberry grower, making the transfer of plants to the field, will have at his disposal the material required for his plantations. He can, therefore, choose the most suitable moment for his planting.

The main drawbacks of micropropagation are economic and psychological in nature. The cost of producing in vitro plants is at present rather high. In our laboratory with a highly qualified staff and a high investment, cost price reached 0.06 US$ per plant at the end of 1974. Industrial equipment and certain improvements now under study could reasonably permit a reduction in cost price.

Nurserymen will have to accept an important transformation of their plantations. Strawberry growers will also have to agree with this new type of plant and to adapt themselves to the few new requirements. As for us, we believe that the difficulties arising out of this new technique are of minor importance as compared to the benefits. Furthermore, this technique has been the object of a patent for our research station.

We conclude by presenting the possible organization of production in an industrial laboratory. The proposed scheme tries to divide the laboratory activity throughout the year, avoiding where possible too much rush of a time.

Mid-February and mid-April production (Delivery from March 15th to April 15th).

— Supply to nurserymen who continue with traditional multiplication for cold storage plant or summer plant.
— Supply to strawberry growers who execute first and brief traditional multiplication at home ($\pm$10 runners/mother plant) for plantation at the end of July and the beginning of August.
— Supply to strawberry growers of everbearing cvs. in the case of production of late strawberries.

May–July production (Delivery in June–July).

— Supply to strawberry growers who plant cold storage plant.

July–September–October production (Delivery in August–October).

— Supply to nurserymen who practice traditional multiplication.
— Supply to amateurs.

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References see page 207.

8. Tissue Culture Studies on Cereals

Y. YAMADA

1. Introduction

The technique of cell and tissue culture has been widely developed, and many publications on growth, metabolism, and differentiation of tissue culture of dicotyledons can be cited. However, studies with monocotyledons, especially the cereal plants, are still relatively few for which there are several reasons.

1. Until 10 years ago there had been very little success in inducing callus and in maintaining good growth of cereal tissue cultures (STRAUS and LARUE, 1954; NORSTOG, 1956; CAREW and SCHWARTING, 1958; TAMAOKI and ULLSTRUP, 1958). The former problem was resolved by using higher concentrations of auxins in the media employed for cereals (YAMADA *et al.*, 1967b; CARTER *et al.*, 1967) than were used for dicotyledonous material. The first successful callus induction of cereal plants was induced from the young endosperm of maize and which required no auxins but only yeast extract (STRAUS, 1954; STRAUS and LARUE, 1954). This peculiarity gave the impression that cereal tissue cultures differed from other plant tissue cultures. Now we realize that callus induction from cereals is easily accomplished by using high concentrations of auxin in the media.

2. It is difficult to obtain a suitable size of homogeneous tissue from cereal plants. Pith tissues in the stem and in the cambium layer of the root have been used for tobacco and carrot cultures respectively, and supply a large amount of homogenous material. But in cereals these areas provide little homogeneous tissue.

3. Cereals are important sources of carbohydrate (starch) in our food supply. In contrast, sugars are required as the source of carbohydrates for their tissue culture. Thus, for practical applications, e.g. the production of carbohydrates, cereal tissue culture is not advantageous.

However, some cereals, such as maize and wheat, have very important uses in genetic studies. For physiological studies, cereals are very attractive material, especially for the study of metabolism and regulation. In fact, the tissue culture studies on cereals reported to date have been mainly in these areas.

2. A History of the Tissue Culture of Cereals

2.1 Maize (Zea mays L.)

The first successful tissue culture among a cereal was raised from maize endosperm (excised 8–18 days after pollination) by LARUE in 1949. These showed slow

general enlargement for nearly a month, then began to proliferate at many points and finally produced a loose, tumorous mass with little differentiation. Since then various workers have undertaken maize endosperm tissue culture using the same method. They found that the in vitro growth of the tissue differed widely with different strains of maize (STRAUS and LARUE, 1954; STERNHEIMER, 1954; TABATA and MOTOYOSHI, 1965). However, the culture media used always contained some natural products. The first synthetic medium for maize endosperm culture was used by STRAUS in 1960, when calli from the endosperm of Black Mexican Sweet maize were maintained by subculturing them on a synthetic medium containing mineral salts, 2% sucrose, vitamins and 1.5×10^{-2}M asparagine. Coconut milk had no appreciable effect on the growth of the tissue, possibly because it contains indoleacetic acid which has been found to inhibit the growth of endosperm tissues. TAMAOKI and ULLSTRUP (1958) used small explants of intercalary meristems from the maize stem and obtained callus, but their subcultures did not grow. Thus, in maize, viable callus has always been obtained from the endosperm.

2.2 Rye (Secale cereale L.)

In 1958, CAREW and SCHWARTING succeeded in obtaining callus from the rye embryo, though the continuous culture of excised rye roots had previously been accomplished by ROBERTS and STREET (1955). In 1970, MULLIN obtained vigorously growing callus from a clone of excised roots of the cv. Black Winter.

2.3 Barley (Hordeum vulgare L.)

NORSTOG succeeded with barley embryo cultures in 1961 and has continued his work on their growth behavior in cultures (NORSTOG, 1967, 1970). In 1968, GAMBORG and EVELEIGH established suspension cultures of barley. They derived their cultures from root sections of seedlings which they cultured in a defined medium of mineral salts, sucrose, B vitamins and 2,4-D with nitrate and ammonia. They reported that in the early period of culture, cell aggregates readily differentiated to form roots, but that this characteristic diminished after several generations of subculture. In 1970, YAMAGUCHI *et al.* reported callus induction from the barley embryo and investigated the most suitable medium for its isolation and the growth of cell suspension.

2.4 Rice (Oryza sativa L.)

Studies of rice tissue culture have been pursued in Japan since the 1960s. FURUHASHI and YATAZAWA (1964) first succeeded in callus induction using rice stem nodes, and emphasized that yeast extract was an essential factor (YATAZAWA *et al.*, 1967). YAMADA *et al.* (1967b) induced rice calli and cultured them subsequently on a synthetic medium. They reported that IAA as well as 2,4-D was capable of inducing callus from the roots and nodes of the shoots (YAMADA *et al.*,

1967a). Furthermore, they succeeded in inducing the redifferentiation of shoots as well as roots, and restored whole plants from rice calli (NISHI *et al.*, 1968). MAEDA (1965) also succeeded in inducing callus formation from rice seedlings on a medium containing 2,4-D and yeast extract, and has actively studied rice tissue culture in terms of varietal differences, histology and differentiation (MAEDA, 1967a, b, 1968, 1969, 1971a, b; NAKANO *et al.*, 1975).

2.5 Oats (Avena sativa L.)

Webster established callus from germinating oat seeds and cultured the chrysanthemum nematode on this callus in 1966. CARTER *et al.* also induced callus from germinating oat seeds in 1967 on a synthetic medium and observed that oat callus induction and growth were very sensitive to the auxin (2,4-D and IAA) concentrations but relatively unaffected by kinetin.

2.6 Wheat (Triticum monococum L., Triticum aestivum L., Triticum dicoccum Schubl)

TRIONE *et al.* (1968) tested 20 different media used for wheat tissue culture and established suspension cultures of cells which were derived from cotyledonary nodes. Simultaneously, GAMBORG and EVELEIGH (1968) succeeded in producing suspension cultures of wheat using a defined medium consisting of mineral salts, sucrose, B vitamins and 2,4-D. In 1969, SHIMADA *et al.* reported wheat callus formation and single cell cultures. SHIMADA (1971) found that the calli of common wheat consisted of eudiploid and aneuploid cells with almost the same frequencies.

2.7 Sorghum (Sorghum vulgare Pere)

STROGONOV *et al.* (1968) succeeded in culturing tissue from sorghum and compared its salt resistance with other cultured tissues. MASTELLER and HOLDEN reported the culture of sorghum callus, the initiation of shoot primodia, and the development of plants, in 1970, from callus of this species.

2.8 Millet (Panicum miliaceum L.)

RANGAN (1974) established a somatic tissue culture on the Murashige-Skoog medium with 2,4-D and coconut milk, and regenerated plantlets from this callus by the method used with rice callus.

2.9 Ryegrass (Lolium perenne L.)

NORSTOG (1956) reported the tissue culture of the endosperm of English rye-grass. He maintained the callus in vitro for more than two and a half years on a medium containing natural products.

2.10 Brome Grass (Bromus inermis Leyss.)

GAMBORG *et al.* (1970) reported a successful suspension culture of brome grass, from callus which had first been initiated by SCHENK and HILDEBRANDT (1972) from the mesocotyl of Canadian brome grass. They also reported the formation of embryos in their cell suspension cultures and the formation of albino plants.

Recently induction of callus and organogenesis has been reported in various cultivars of maize, wheat, rye and sorghum by MASCARENHAS (1975a, b), and NAKANO *et al.* (1975) have obtained complete regeneration of plants from endosperm callus of rice.

3. Media for Callus Induction and Cell Culture

Media for callus induction and cell culture of cereal plants have been widely studied. Table 1 shows the main media used for callus induction and for the subculture of barley, maize, millet, oats, rice, rye, sorghum and wheat cells. Looking at these media, it is surprising that natural products, i.e. yeast extract, coconut milk or casein hydrolysate, have been used as nutrients in all but a few. The use of natural products in the media has severely restricted the type of experiment to be performed with the tissue because they contain unknown factors, and the quantities of the known and unknown factors vary with each group. With one maize strain culture, used in studies of the biosynthesis of anthocyanin (STRAUS, 1959), it soon became clear that a synthetic medium was needed, and a concentrated effort was made towards its development (STRAUS, 1960). Recently, SCHENK and HILDEBRANDT (1972) reported a synthetic medium (Table 2) for the induction and growth of monocotyledonous plant cell cultures. The growth of various grasses and cereal cells on it is presented in Table 3. They concluded, as had YAMADA *et al.* (1967a; YAMADA, 1969), that high levels of auxin-type, growth-regulating substances generally favored the cell culture of monocotyledonous plants. In the future tissue culture of cereal plants, the use of natural complex mixtures in the media should be avoided.

As the trigger compounds for callus induction (cell division), auxins are essential for most plant tissues. The most effective auxins are synthetic, 2,4-D and NAA. IAA is a natural auxin which is usually required in a higher concentration than a synthetic auxin in the medium for callus induction (YAMADA, 1967a, b). This phenomenon is thought to be due to the fact that the absorbed IAA in tissues may be changed metabolically to an inactive form in the cells (GALSTON *et al.*, 1953). This coincides with reported results for the tissue culture of cereal plants. 2,4-D is the most common and effective auxin for inducing callus and subculturing the cells of cereal plants. Furthermore, the concentration of 2,4-D used for callus induction with these plants tends to be higher than that for callus formation from common dicotyledonous plants. In the case of IAA, 100 mg/l was required for callus induction from rice nodes (YAMADA *et al.*, 1967b) and from oats (CARTER *et al.*, 1967). This suggests that more of the incorporated IAA is converted to the ineffective form in the monocotyledonous cereals than in dicotyle-

Table 1. The media for callus induction from cereal plants

Cereals	Plant part	Auxin	Medium	Natural nutrients	Culture condition	References
Barley *Hordeum vulgare* L.						
cv. Ehimehadaka 1	embryo	2,4-D, (3 mg/l)	Murashige-Skoog	yeast extract	liquid	YAMAGUCHI *et al.* (1970)
cv. Gateway	root	2,4-D, (2 mg/l)	PRL-4 (induction) B-5 (sub-culture)	none	agar (induction) liquid (sub-culture)	GAMBORG and EVELEIGH (1968)
Maize *Zea mays* L.						
cv. Surprise Sweet, cv. Black Mexican Sweet	endosperm	none	White (major elements) Nitsch (minor elements)	yeast extract	agar	STRAUS and LARUE (1954)
cv. Black Mexican Sweet	endosperm	none	White (major) Nitsch (minor) vitamins Asparagine	none	agar	STRAUS (1960)
Millet *Panicum miliaceum* L.						
cv. RPMC-15	mesocotyl	2,4-D, (10 mg/l)	Murashige-Skoog	coconut milk	agar	RANGAN (1974)
Oat *Avena sativa* L.						
cv. Sun II	germinating seed	IAA, (2 mg/l) + NAA, (25 mg/l) or 2,4-D, (5 mg/l)	Heller	none	agar	WEBSTER (1966)
cv. Victory	germinating seed	IAA, 1.4×10^{-3}M or 2,4-D, 2.2×10^{-5}M	Linsmaier-Skoog	none	agar	CARTER *et al.*, (1967)

Table 1 (continued)

Cereals	Plant part	Auxin	Medium	Natural nutrients	Culture condition	References
Rice *Oryza sativa* L.						
cv. Kinmaze	root	2,4-D, (2 mg/l)	Heller glycine, tryptophan, thiamine, nicotinic acid, pyridoxine	yeast extract	agar	YATAZAWA *et al.* (1967)
cv. Kyoto Asahi	germinating seed, roots, shoot node	2,4-D, (5 mg/l) or IAA, (100 mg/l)	Murashige-Skoog	none	agar	YAMADA *et al.* (1967)
cv. Norin 16	germinating seed	2,4-D, (2 mg/l)	R-2	none	liquid	OHIRA *et al.* (1973)
Rye *Secale cereale* L.						
cv. Canadian Spring	embryo	2,4-D, (1 mg/l)	Heller modified	yeast extract	agar	CAREW and SHWARTING (1958)
cv. Black Winter	root	2,4-D, (6 mg/l)	White myo inositol	yeast extract	agar	MULLIN (1970)
Sorghum *Sorghum vulgare*						
Pere (*bicolor* Moench.)	root and tillering node	2,4-D, (5 mg/l)	Murashige-Skoog Ca-pantho-thenate	casein hydrolysate	agar	STROGONOV *et al.* (1968)
cv. Norghum and North Dakota 104	shoot primodia	2,4-D, (5-15 mg/l)	Murashige-Skoog	coconut milk	agar	MASTELLER and HOLDEN (1970)

Table 1 (continued)

Cereals	Plant part	Auxin	Medium	Natural nutrients	Culture condition	References
Wheat *Triticum*						
T. monococcum L., *T. vulgare* Vill. cv. Thatcher	root	2,4-D, (2 mg/l)	PRL-4 (induction) B-5 (subculture)	none	agar (induction) liquid (subculture)	GAMBORG and EVELEIGH (1968)
T. aestivum L. em. Thell. cv. Chinese Spring	root tip	2,4-D, (5 mg/l)	White	none	agar	SHIMADA (1971)
T. dicoccum Schubl. cv. Hokudai	ovaries	—	—	—	—	—
T. aestivum L. em. Thell. cv. Chinese Spring, cv. S-615	germinating seed, stem	2,4-D, (0.5-2 mg/l)	White	casein hydrolysate or coconut milk	agar	SHIMADA *et al.* (1969)
T. dicoccum Schubl. cv. Hokudai	germinating seed	—	—		—	—
T. monococcum L. var. flavescens	germinating seed	—	—	—	—	—
Rye grass *Lolium perenne* L.	endosperm	IAA, (1.0 mg/l)	White	yeast extract or coconut milk	agar	NORSTOG (1956)

Table 2. Composition of SH medium (SCHENK and HILDEBRANDT, 1972)

	SH medium
Major elements	
KNO_3	2500 mg/l
$MgSO_4 \cdot 7H_2O$	400
$NH_4H_2PO_4$	300
$CaCl_2 \cdot 2H_2O$	200
Minor elements	
$MnSO_4 \cdot H_2O$	10.0
H_3BO_3	5.0
$ZnSO_4 \cdot 7H_2O$	1.0
KI	1.0
$CuSO_4 \cdot 5H_2O$	0.2
$NaMoO_4 \cdot 2H_2O$	0.1
$CoCl_2 \cdot 6H_2O$	0.1
Iron compounds	
$FeSO_4 \cdot 7H_2O$	15
Na_2EDTA	20
Organics	
Inositol	1000
Thiamine · HCl	5.0
Nicotinic acid	5.0
Pyridoxine · HCl	0.5
Growth-regulating substances	
2,4-D	0.5
*p*CPA	2.0
Kinetin	0.1
Sucrose	30[a]
Agar	6[a]

[a] g/l.

Table 3. Growth of various cereal tissue cultures (SCHENK and HILDEBRANDT, 1972)

Species	Cultivar	Growth rate index[a] in darkness
Barley (*Hordeum vulgare*)	Himalaya	4
Timothy (*Phleum pratense*)	Old Gold	5
Brome grass (*Bromus inermis*)	Manchar	3
Wheat (*Triticum* sp.)	Vernal, Gaines,	3
	Justin, Khapi	2
Oat (*Avena sativa*)	Tetraploid C.I. 7232	3
Rice (*Oryza sativa* L.)	Tai N. 1, IR-8	2
Triticale (*Triticum* × *Secale*)	C.I. 11, 504	3
Triticum monococcum		3
Grain sorghum (*Sorghum* sp.)	Pioneer Hybrid 7480	3
Rye (*Secale cereale*)	Tetrapetkus, Gator	2
Corn (*Zea mays*)	SC 313 inbred	1
Orchard grass (*Dactylis glomerata*)	Potomac	3

[a] Growth index: 5 = excellent growth; 1 = poor growth.

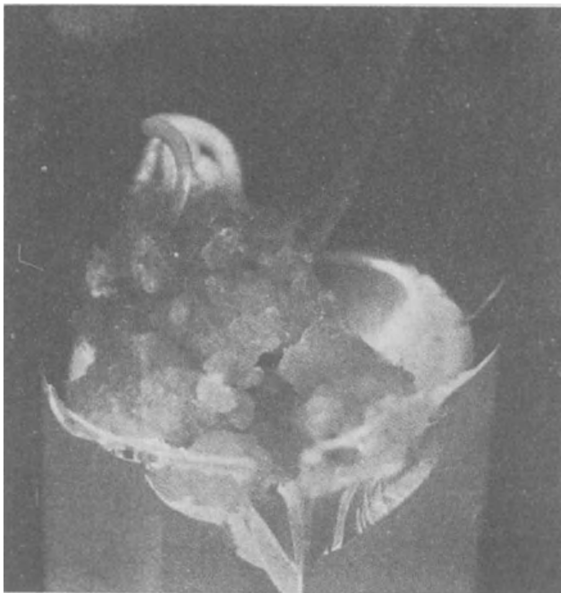

Fig. 1. Callus induction from rice seeds using Murashige-Skoog basal medium containing 2,4-D 10 mg/l (unpublished)

donous plants. Tables 4 and 5 show the effect of growth regulators on callus induction in rice and wheat respectively. Both types of callus were formed at a high 2,4-D or IAA concentration, regardless of the kinetin concentration. At low concentrations of 2,4-D, a high kinetin concentration seems to enhance callus induction in rice. However, this may be due to the activation of callus growth by kinetin after the initiation of callus by auxin, since the growth of callus in the subcultures showed a strong correlation with the concentration of kinetin. The use of IAA in the same concentration range as 2,4-D produced no callus formation. At 50 and 100 mg/l of IAA, respectively, callus induction from both wheat and rice occurred. The obvious conclusion from these data is that auxin, and not kinetin, is absolutely essential to induce callus from rice and wheat tissues. The other cereals also seem to show the same response to auxins, the only exception being callus induced from the maize endosperm. With the maize endosperm, no exogenous auxins were added to the media but very healthy calli were obtained (LARUE, 1949; STRAUS and LARUE, 1954; STERNHEIMER, 1954; TABATA and MOTOYOSHI, 1965). The important point in producing this endosperm callus is that very young endosperm, taken 12 days after pollination (STRAUS and LARUE, 1954) has always been used. Cell division in the endosperm is more or less generalized throughout the mass up to 7–8 days after pollination, after which the outermost layer of cells produces most of the new cells on its inner side. Therefore, at 12 days post-pollination cells produced by the peripheral layer have a meristematic appearance (STRAUS, 1954). From these observations, it seems that there must be sufficient production of trigger compounds, such as IAA, for the activation of cell division of the endosperm at 12 days post-pollination. Thus, the endogenous

Table 4. Growth of rice callus in subculture. The original callus was induced from a medium containing 2,4-D (2 mg/l) and kinetin (1 mg/l). Inoculated callus weight; 210–220 mg, Incubation for 90 days. (YAMADA *et al.*, 1967a)

	mg/l	Kinetin				
		0	0.01	0.1	1.0	5.0
2,4-D	0	254	251	280	269	255
	0.1	1370	1521	1261	1650	1820
	0.5	1279	1133	1268	1676	1350
	2.0	1222	1594	1333	1650	1312
	10.0	1209	1326	1274	248	261
IAA	0.1	248	271	254	243	250
	0.5	258	330	269	236	245
	2.0	300	282	1170	970	238
	10.0	245	1495	228	238	245

Table 5. Effects of 2,4-D and kinetin on callus induction in three groups of wheat (SHIMADA *et al.*, 1969)

Material	Kinetin (mg/l)	2,4-D (mg/l)					
		0	0.1	0.5	1.0	5.0	10.0
T. aestivum	0–0.02	–	–	±	++	++	+
	0.2	–	±	±	+	+	+
	0.4	–	–	+	+	+	+
T. dicoccum	0–0.2	–	–	×	+	+	+
	0.4	–	–	×	±	+	+
T. monococcum	0	–	–	×	++	+	+
	0.02–0.4	–	–	×	+	+	+
	Kinetin (mg/l)	IAA (mg/l)					
		0	1	5	10	50	100
T. aestivum	0	–	–	–	±	+	*
	0.01–0.1	–	–	–	–	+	*
	0.2	–	–	–	–	±	*

Note: × : not tested, – : no callus formation, ± : occasional callus formation, + : consistent callus formation, ++ : large callus formed, *: no seed germination.

auxin(s) produced in the endosperm appears to induce callus by explantation of the endosperm. YAMADA *et al.* (YASUDA and YAMADA, 1970; YAMADA and YASUDA, 1971; YAMADA *et al.*, 1971a, b) have mainly studied the mechanism of callus induction from dicotyledons. They reported that exogenously incorporated 2,4-D interacts (binds) with proteins, based on the formation of 2,4-D/lysine rich histone complexes during the early stage of callus induction. An interesting question is whether endogenously produced auxin in the maize endosperm acts on basic proteins in the nucleus of the cell to produce cell division.

4. Genetic and Physiological Studies with Cereal Tissue Cultures

4.1 Genetic Work

Maize and wheat are the most important cereal crops and are suitable for genetic studies. In 1954, STRAUS reported that mature cells of the cultured endosperm of maize are frequently multinucleate, polyploid, and often possess extremely lobed nuclei of bizarre appearance. He also observed hypoploid cells and mitotic abnormalities which were manifested by the presence of many chromosome bridges and lagging chromosomes. Various other workers have studied the interrelationships of the in vitro growth of tissue using different cultivars of maize (where the starchy, waxy, and sugary types may be related genetically), to the ease of callus induction. STRAUS and LARUE (1954) reported that of 15 cultivars of maize tested, only two sugary cultivars gave a growth response. STERNHEIMER (1954) mentioned that although none of the waxy and starchy endosperms that had been isolated from many cultivars grew under in vitro conditions, the sugary types could be successfully cultured. TAMAOKI and ULLSTRUP (1958) reported that of 25 inbred lines of maize and 32 of their single crosses, only four from sugary-type endosperms showed continuous growth in successive transfers, whereas subcultures of the waxy and starchy endosperm tissues showed only limited growth. However, TABATA and MOTOYOSHI (1965) found that of four inbred strains (2 starchy, 1 waxy and 1 sugary) of maize tested, callus was formed only in endosperm from the starchy strains. They suggested that endosperm cells with certain genetic factors and a specific physiological state are activated by amino acids in the medium, so the cells multiply in vitro. As a conclusion, the ease of callus induction from maize endosperm is not directly controlled by the waxy or sugary gene but other gene(s) must be involved. Recently, SHERIDAN (1975) has initiated maize tissue culture and believes that the successful development of a tissue culture system for maize, for which the culture and selection methods commonly employed by microbial geneticists might be used, could contribute to a greater utilization of these species in basic genetic and developmental studies. He has determined that the first step in the establishment of such a system is to characterize the basic parameters of the induction and growth of callus from sporophyte tissue. With rice, YAMADA *et al.* (1967b) reported that chromosomal aberrations were observed in calli induced from the roots and nodes of shoots. The frequency of aneuploid cells was 80% and 57% in the calli from the roots and nodes, respectively.

SHIMADA *et al.* (1969) observed that the calli of common wheat consisted of eudiploid and aneuploid cells at almost the same frequencies and that the great majority of aneuploid cells had 42 ± 3 chromosomes. KAO *et al.* (1970) described cell suspension cultures of *Triticum monococcum* and *T. aestivum* with changes in their chromosome numbers and abnormal karyotypes. However, SHIMADA (1971) reported that, in common wheat, the chromosome constitution of callus cells derived from root tips subcultured for two to four years was similar to that of callus cells from the ovary; in both diploid cells predominated.

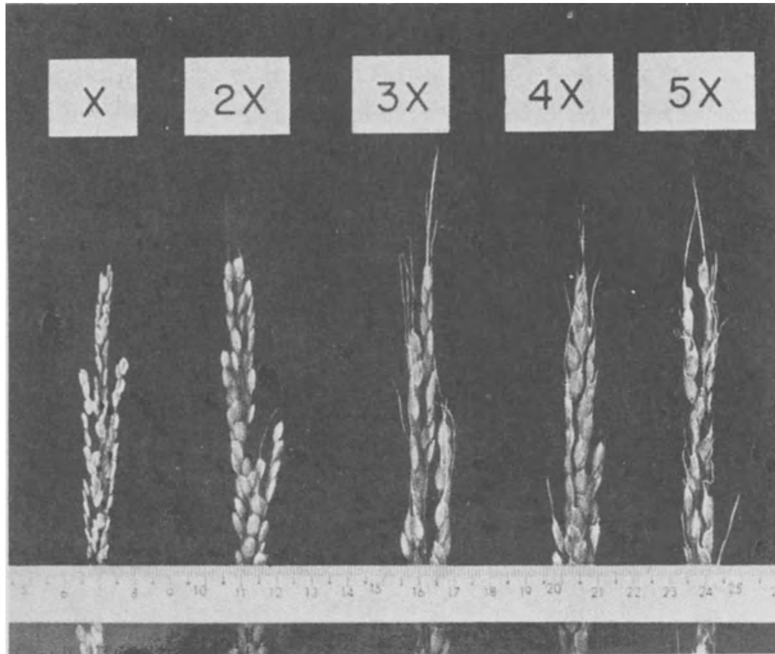

Fig. 2. Ears of various ploidy plants produced from anther and ovary cultures of rice. (Courtesy of Dr. T. NISHI)

In vitro induction of haploids is of great importance in cereal breeding, as homozygous plants can be obtained in a single generation, and also the mutants can be easily detected (see Chap. II.1 of this Vol.). NIIZEKI and OONO (1968, 1971) first succeeded in the regeneration of haploid plants from excised anthers of rice. Since then haploid tissue and plants have been obtained in wheat, barley, rye, and maize (see Chap. II.3 of this Vol., Table 2). In addition haploids have also been induced in barley (KASHA and KAO, 1970) and wheat (BARCLAY, 1975) by the bulbosum technique in which chromosomes of one parent are eliminated during culture of a hybrid embryo. For details of this method see Chapter II.4 of this Volume.

The isolation, culture and fusion of protoplasts has also become a very attractive subject because of its potential in plant improvement (COCKING, 1973; BAJAJ, 1974; see Chap. IV.1 of this Vol.). As the standard techniques are worked out the current areas of applicability should be widened.

Cereal protoplasts have been isolated from coleoptile and primary leaves of *Avena* (RUESINK and THIMANN, 1966), and from the root tips of various cereals (POWER *et al.*, 1970). EVANS *et al.* (1972) were able to isolate protoplasts in quantity from the mature leaves of wheat, barley and rye. Subsequently MOTOYOSHI (1972) and SCHASKOLSKAYA *et al.* (1973) obtained protoplasts from maize callus and barley mesophyll cells respectively. Though up till now there has been no report on the successful culture of cereal protoplasts, more positive results are shortly expected as a result of the wide interest in this field.

4.2 Physiological Studies

There are very few studies on the metabolism and physiological regulation of cultured cereal cells, and these are mainly concerned with cultured cells of rice.

GAMBORG and EVELEIGH (1968) demonstrated the presence of several glucanases in the suspension-cultured cells of wheat and barley and in the culture medium. The presence of a laminaranase (end-β-(1→3)-D-glucan-glucanohydrolase) that did not attack lichenan was established. Their culture media for wheat contained an oligosaccharide which, on acid hydrolysis, gave galactose, arabinose and xylase. SAKA and MAEDA (1973) studied the α-amylase isoenzyme in rice callus grown on media containing various carbon sources. They found six isozymes, whose activities changed in compliance with the total α-amylase activity. IGAUE *et al.* (1973) studied the syntheses of the different types of intracellular soluble acid phosphatase isozymes which occur in rice cell cultures in response to the phosphate concentration of a medium and suggested that a phosphate-phosphatase isozyme regulatory system plays an important role in their phosphorus metabolism. FURUHASHI and YATAZAWA (1970) reported the interaction of methionine, lysine, threonine and isoleucine in the amino acid requirements of rice callus and proposed that a possible regulatory mechanism works in the amino acid nutrition of this type of tissue and they also investigated the arginine metabolism and ornithine cycle (FURUHASHI *et al.*, 1972; LEE *et al.*, 1972). Regarding the amino acid incorporation system in maize endosperm cultured tissue, GRAEBE and NOVELLI (1966) reported that the system exhibits the established requirements for protein synthesis and is sensitive to ribonuclease and puromycine, but not to deoxyribonuclease or chloramphenicol.

In surveying alkaloid production with rye callus, CHANG and CAREW (1968) used static cultures as the host for clavine alkaloid production. When this rye callus was present in a liquid medium which had been inoculated with Hofmann's Pennisetum, strain 231, there was an increase in alkaloid accumulation over that found when strain 231 was cultured in a medium without callus.

In the literature on physiological studies, there are several reports on nutritional requirements (YATAZAWA and FURUHASHI, 1968; GREEN *et al.*, 1974; OHIRA *et al.*, 1975); the composition of cultured cells (YAMADA *et al.*, 1967a; GAMBORG and FINLAYSON, 1969; ADACHI and KATAYAMA, 1969; WU and LI, 1971); cytological observations (MAEDA, 1971a, b); and metabolic changes (GAMBORG and LARUE, 1968; TOKUDA and YAMAGUCHI, 1972).

5. Organ Redifferentiation and Regeneration

The redifferentiation of shoots and roots, and also the regeneration of whole plants from rice callus was the first successful work on cereals. NISHI *et al.* (1968) cultured callus derived from the roots of rice on the Linsmaier-Skoog medium (1965) containing 10^{-5}M 2,4-D. This callus when transferred to the same medium but without auxin, and incubated in the light, redifferentiated to form both

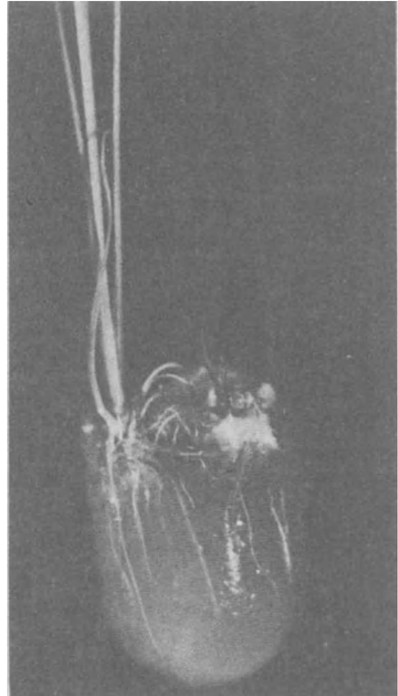

Fig. 3. Redifferentiated shoots and roots in the second transfer of rice callus cultured on a medium without auxin (After NISHI *et al.*, 1968)

shoots and roots, and subsequently whole plants. The regenerated plants were diploid, though a few showed a peculiar phenotype. Thus, they concluded that auxin is one of the most significant key-regulators for differentiation and redifferentiation in monocotyledonous plants. CARTER *et al.* (1967) also reported that oat callus showed redifferentiation into a large number of shoots and a few roots when the callus was transferred to an auxin-free medium.

SHIMADA *et al.* (1969) reported that wheat callus showed almost constant root formation and occasional shoot production on media containing low concentrations of auxin. GAMBORG and EVELEIGH (1968) also observed that wheat and barley suspension cultures produced roots when 2,4-D was replaced by IAA or NAA. MASTELLER and HOLDEN (1970) reported that when sorghum callus was transferred to a medium in which NAA had been substituted for 2,4-D and cultured in light, it soon turned green and buds were observed. Recently RANGAN (1974) reported that when cultures of millet were transferred to an auxin-free medium, shoot bud initiation was evident within weeks. Similarly, an auxin used at reduced concentrations also induced shoot buds.

Table 6 shows the auxin concentrations used in the media for redifferentiation and regeneration. For callus culture, all the media contain 2,4-D, which indicates that the cereals require the strong action of an auxin to induce callus and to maintain their growth in subcultures. However, for organogenesis, it is essential that there be a decrease in the 2,4-D concentration or the substitution of a weaker

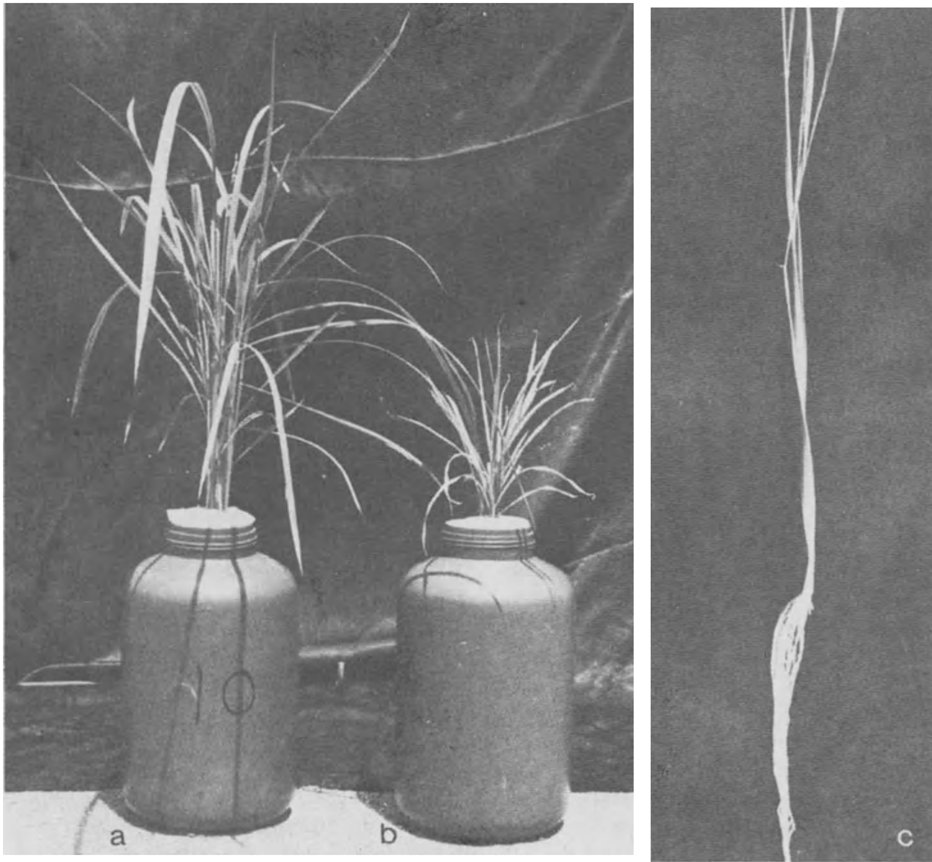

Fig. 4a–c. Peculiar phenotypes in restored MCl plants. (a) normal restored phenotype; (b) dwarf phenotype; (c) twisted phenotype. After NISHI *et al.* (1968)

auxin, such as IAA or NAA. So it is obvious from these studies that organ redifferentiation or regeneration in cereals is initiated only when auxin level is low.

6. Conclusions

The techniques for the callus induction, cell culture, redifferentiation and regeneration of cereal plants have been fairly well established. The culture of cereal plant cells, however, does not seem to have an immediate application toward increasing the world's supply of carbohydrates, although it holds great promise for producing new types of cereal plants by anther cultures and protoplast fusion. For genetic modifications and the selection of mutants, cultured haploid cells and

Table 6. Auxins used in redifferentiation media for cereal plants

Cereals	Condition of auxin		Redifferentiation	Reference
	Cell culture	Organ formation		
Oryza sativa L.	2,4-D, 10^{-5}M	no auxin	shoot and root regeneration	NISHI *et al.* (1968)
Avena sativa L.	2,4-D, 2.3×10^{-5}M $\sim22.5\times10^{-5}$M	no auxin	many shoots and few roots	CARTER *et al.* (1967)
Triticum aestivum L.em. Thell.	2,4-D, 1 mg/l	2,4-D less than 1 mg/l	many roots and few shoots regenerated	SHIMADA *et al.* (1969)
Triticum vulgare	2,4-D, 5×10^{-6}M	IAA 5×10^{-6}M	many roots	GAMBORG and EVELEIGH (1968)
Hordeum vulgare L.	2,4-D, 10^{-5}M	IAA 5×10^{-6}M	many roots	
Sorghum bicolor Moench	2,4-D, 5 mg/l	NAA 5 mg/l	regeneration	MASTELLER and HOLDEN (1970)
Panicum miliaceum	2,4-D, 10 mg/l	no auxin or reduced conc. of auxin	bud formation	RANGAN (1974)

somatic cells are most efficient and it is now possible to regenerate whole plants from these cultured cells. New cereal plants which fix the nitrogen, and cereal varieties with strong disease resistance may one day be obtained by genetic manipulation, and in future may cause a revolution in the world's basic food supply. Furthermore, as basic research on the cereals is broadened, a wide variety of applied studies with open-ended applications, should be possible.

References see page 207.

9. Ovule Culture: Fundamental and Pragmatic Research for the Cotton Industry

C. A. BEASLEY

1. Introduction

Compared to the extensive research on seed quality and germination, developmental physiology of seed formation has received little attention. This seems particularly true for cotton, despite the fact that fibers are integral components of ovules (seed) and that fibers are dependent in their development upon the ordered progression of fertilization, embryo growth and seed maturation. With the advent of plant tissue and organ culture, the developmental seed physiologist was furnished with new research tools with which to seek both fundamental and practical information. However, as for many species, procedures specific to cotton were not available (e.g., methods for the suitable culture of parent plants, harvest of flowers, and aseptic transfer of immature seed to a supportive growth medium).

It has long been known that certain plant growth regulators markedly alter the time of initiation as well as the subsequent development of reproductive organs. These substances also affect the division and elongation of cells and the subsequent formation of their secondary walls. Thus, concomitant with recognition of the need for establishing specific methodology, it was fully realized that proposed research must be concerned in detail with determining the effects of a number of phytohormones on the in vitro growth of cotton ovules and their associated fiber. It was conceivable then, that from information obtained via in vitro studies, the number, length, and thickness of cotton fibers might be improved in the field by application of specific plant growth substances. Despite these theoretical possibilities, it was realized that increases in yield of seed and fiber (via pragmatically judicious and precisely timed application of plant growth substances) would, in all probability, be dependent upon (1) new and more determinant cultivars, and (2) greater numbers of plants per acre (this also promoting a more determinant growth habit). These adjustments in cotton culture would provide the required decrease in the time over which bolls (ovaries) are set, and improve the potential for precisely timed application of appropriate growth regulators. In anticipation of these advances in plant breeding and in narrow-row production practices, development of procedures for the in vitro culture of cotton ovules was begun.

2. Salient Features of Developmental Morphology

Anatomic, morphologic, and physio-agronomic characteristics of cotton have been described in a voluminous literature. The following are a number of impor-

tant writings recommended for the interested reader: HAYWARD (1938), LANG (1938), O'KELLEY and CARR (1953), THARP (1960), JOSHI *et al.* (1967), MAUNEY (1968), CARNS and MAUNEY (1968), JENSEN (1968), LINTILHAC and JENSEN (1974). This article does not intend to undertake a comprehensive review; rather, the references used above and observations from this laboratory form the basis for a limited treatment of flower, seed, and fiber development. This brief presentation is intended to aid the reader in understanding (1) the reason for originally undertaking the work presented, and (2) the intracacies of harvest, subtransfer and culture procedures.

At the base of each mainstem leaf on a cotton plant there are normally two axillary buds. The first and most prominent gives rise to either a vegetative or fruiting branch. If the first axillary is aborted, or if the plant is growing vigorously, the second axillary bud will usually give rise to a vegetative branch regardless of the morphology of the first. Floral branches are initiated on about the 7th–9th node above the cotyledonary leaves. The interval between the appearance of new fruiting branches is about three days; between the successive nodes of the fruiting branch—each with a floral bud (square),—the interval is about six days. Approximately 25 days are required, after the square is first visible, for the flower bud to undergo anthesis. Flowering is, thus, progressive and for a time it becomes more rapid as the plant grows. The maximum rate of increase in floral bud length occurs during the 24 h preceeding anthesis. Anthesis itself occurs at about daylight and approximately 90% of the flowers open within an hour. Multipored pollen grains germinate in about $^1/_2$ h after their deposition on the stigmatic hairs. Fertilization is accomplished, for most ovules, by the end of the first day postanthesis. After flowering and boll setting have continued for a number of weeks, both plant growth and flower production decrease and may stop altogether. Also, many (if not most) of the floral buds abort before anthesis. Typically, early fruiting varieties of cotton shed most late buds and bolls, whereas late fruiting varieties not only bloom later but set and retain bolls over a longer period of time. Commercial cottons produce marketable lint fibers (varying between species and cultivars, but approximately 2.5 cm in length) and shorter thickenend fuzz fibers of limited value. Both types of fibers originate as outgrowths (fiber initials) of epidermal cells of the ovule. All epidermal cells (excluding stomatal guard cells and the cells comprising the micropyle) are potential fibers. Lint-fiber initials begin their elongation phase at anthesis, the exact timing of initial elongation varying somewhat between species and cultivars. Lint fiber elongation ceases by 24–28 days postanthesis, and by 50–70 days postanthesis fibers are mature, exhibiting thickened secondary walls (consisting of about 94% cellulose) and spiral twisting. It is generally accepted that fuzz fibers initiate growth in several waves between 4 and 12 days postanthesis. Both the range in time of initiation and the extent of fuzz fibers formed vary between species and cultivars. Concommitant with the time of lint fiber maturity, the boll wall splits and opens along locular suture lines. Certain of these developmental features, as viewed with a scanning electron microscope, have been presented elsewhere (BEASLEY, 1975; STEWART, 1975).

3. Basic Cultural Procedures

3.1 Glasshouse Production of Parental Material

Unless otherwise noted, all experimental cotton has been *Gossypium hirsutum* var. Acala SJ-1. As research needs dictate (currently, every three weeks) 200, 3.78 l (one gal) plastic pots are prepared containing equal portions of washed concrete sand, sandy loam soil, and sphagnum peat moss. Five delinted seed (inspected to reject damaged or undersized seed) are planted per pot. Almost all seedlings emerge on the third to fourth day after planting. By two weeks after planting, all seedlings but one are removed, selection being made for uniformity of cotyledon size and height above the surface of the soil mix. In the morning, plants are lightly watered (depending on need); in the afternoon, they are more heavily watered. Hoagland's-type nutrient solution is administered each week; three days subsequent to application of this complete fertilizer, a KNO_3 fertilizer solution is applied. Concomitant with each 3-week planting, a set of two hundred plants (140 days postplanting) is discarded. To eliminate excess shading and a "bench effect" as much as possible, plants of each set are moved (as well as randomly relocated within a bench set) so that younger (shorter) plants receive maximum light exposure. Older (taller) plants are situated to the rear.

Heaters and coolers are automatically activated at 6:00 a.m. and 8:00 p.m. respectively to maintain an alternating temperature regime of approximately 30° C day and 20° C night. Under most ambient conditions, relative humidity reaches a low of 40—45% during the day and a high of 80–85% at night. As need dictates, insecticides are applied (usually at two-three week intervals). By spraying late in the day, rupture of pollen grains and, thus reduced fertilization are precluded, and direct contact with insecticide solution(s) by persons collecting flowers is avoided.

3.2 Methods of Organ Harvest and Aseptic Transfer

First bloom for each set of plants occurs about 60 days postplanting. Peak bloom for each set (total flowers per day) occurs about 6 days later with flower production gradually decreasing after that. To avoid production of flowers deep within the canopy, entire floral branches are removed on the day their first flowers or developing bolls are harvested for their appropriate research purpose. This termination of flowering branches avoids the possibility that "secondary" flowers would have altered responses in vitro or that excessive boll load would reduce extent of flowering.

Anthesis begins about sun up and approximately 90% of the flowers that will open on a particular day do so within a 1 hour period. As a matter of convenience, open flowers are collected (unfertilized ovules) or tagged at 8:00 a.m. If fertilized ovules are desired, tagged flowers are gently tapped at their base to dislodge excess pollen from the dehisced anthers. Most ovules will have been fertilized by the end of the 1st day postanthesis; however, to ensure fertilization, flowers are collected 48 h postanthesis.

Bracts (collectively termed the involucre), fused sepals, and petals are removed from the ovaries containing either fertilized or unfertilized (depending on experiments to be performed) ovules. Ovaries are soaked in a solution of 20% Purex (6.0% NaOCl) and 0.05% Tween-20 (polyoxyethylene sorbitan monolourate—a surfactant added to promote wetting by the disinfectant solution). Ovaries are rinsed 3–4 times with sterile distilled water and are then kept moist by maintaining them in contact with sterile wet towelling until each ovary is momentarily dipped in 95% ethyl alcohol, flame sterilized, and opened. Using aseptic techniques, performed in a filtered-air, positive-flow transfer hood, ovules are gently prodded into a $1^1/_2$ cm-wide, spoon-shaped spatula by breaking the funiculus at its junction with the central placental column. The spatula, with ovules, is gently lowered into the sterile liquid medium as the culture flask is slanted about 45°. The ovules float free and the spatula is removed from the flask. Each 125 ml longneck culture flask, closed with a chrome Kaput, contains 50 ml of liquid medium and about 28–32 ovules from a single ovary (damaged or undersized ovules are rejected, when possible, during the transfer). Certain aspects of the culture procedures just described have been presented in more detail (BEASLEY, 1974).

3.3 Preparation of Culture Media and Quantitative Procedures

The basal culture medium (its composition to be discussed later) is routinely autoclaved (121 C, 20 psi, 15 min) in culture flasks prior to adding supplements of various hormones. Stock solutions of 3-indoleacetic acid (IAA), gibberellic acid (GA_3), 6-furfuryl aminopurine (Kin), and R-S abscisic acid (ABA) are prepared in liter volumes, dispensed into 2–5 ml aliquots and frozen until subsequent use. All additions to the basal nutrient medium are sterilized through 0.22 μ filters and buretted into the appropriate treatment flask prior to transferring ovules. Culture conditions are $33°\,C \pm 1°\,C$ and constant darkness except for occasional brief examination periods. Each treatment normally consists of 8 flasks. Allowing for occasional contamination or abnormal callus formation, at least 5 flasks per treatment are utilized for data recording. A two-week culture period is considered standard for most studies of hormone effects on fiber production; length of culture may be varied however, depending on the data sought. For studies involving sequential hormone applications, extent of fiber production was determined (1) at the end of 14 days from original transfer, and (2) for duplicate treatments at the end of 14 days plus the number of days of preculture. Termination of all treatments at the end of 14 days from original transfer (irrespective of length of preculture for certain treatments) was adequate to demonstrate significant effects of preculture. Thus, all data reported herein are from experiments terminated two weeks postanthesis.

Total fiber development is assessed by determining colorimetrically the amount of dye absorbed by fibers (hereafter referred to as the stain-destain method). Briefly, the method is as follows: (1) 20 ovules (all from a single ovary) with associated fibers are placed for 15 sec in 80 ml of 0.018% toluidine blue 0, (2) nonabsorbed dye is removed by a 60-sec running-water wash, (3) absorbed dye

is removed by 100 ml of destaining solution (1 part glacial acetic acid, 9 parts 95% ethanol), and (4) absorbance of the destaining solution is then determined after one h destaining. Absorbance values are used as a measure of fiber development and are expressed in terms of total fiber units; one 00 unit at 624 nm has been assigned the value of one TFU. Dry weights of ovules and their associated fibers, pooled by treatments, are often determined after recording TFU. The stain-destain procedures have been described in detail (BEASLEY *et al.*, 1974a).

4. Review of Major in vitro Responses

Although ovaries of several plant species have been cultured in vitro (NITSCH, 1963; BAJAJ, 1966), attempts by several workers to culture the isolated ovary of cotton have been unsuccessful. Culture of the isolated cotton ovule first met with only limited success; fiber development was not achieved and ovule growth was for the most part abnormal (JOSHI, 1960). The work with ovules cultured in vitro was expanded and the effects of hormones, casein hydrolysate, and yeast extract on development of embryo and integuments was described (JOSHI and JOHRI, 1972).

First attempts in this laboratory to grow fibers from ovules in culture employed several variations of low-salt, liquid medium and intentionally-submerged immature seed. Similar media solidified with agar were equally unsuccessful. Inundated ovules failed to grow or produce any fiber; agar-based media provided for callus formation and subsequent tissue browning. Numerous agars were employed with both low- and high-salt media; Difco-Bacto provided for more normal growth (less callus and limited fiber production) than did the Noble agars. Reduction of calcium salts, as constituents of the basal medium, and lowering of agar percentage from 1.0 to 0.5 improved normalcy of ovule and fiber development. However, it was not until fertilized ovules (harvested two days postanthesis) were floated on a high-salt, liquid media (MURASHIGE and SKOOG, 1962) that extensive fiber development was observed (BEASLEY, 1971). It was demonstrated that isolated fertilized cotton ovules could be maintained in culture to the point of germination (BEASLEY *et al.*, 1971). Another laboratory (EID *et al.*, 1973; DE LANGHE, personal communications) reported the culture of 5- and 10-day postanthesis ovules that also produced germinable embryos and normal seedlings in vitro.

Our experimentation led to two major modifications of the Murashige-Skoog medium; namely in sugar and nitrogen sources. It was learned that glucose and sucrose provided equal amounts of fiber, with the former causing less browning of fiber and ovules. Fructose alone was an inadequate source of carbon for fiber growth but did not limit ovule size. We also observed that better growth, with less callus and browning, was obtained with KNO_3 than with NH_4NO_3 or most combinations of the two nitrogen sources. Thus, a satisfactory medium was described (BEASLEY and TING, 1973): the sugar source employed was 100 mM glucose plus 20 mM fructose; the nitrogen source was 50 mM KNO_3. Minor modifications were made in other constituents such that concentration could be simply expressed in molarity. Even that medium, selected as standard at the time, has

Table 1. Basal culture medium for cotton ovules [a]

Stock No.	Component	g/l (Stock)	ml Stock/l (Final)	mg/l (Final)	mM (Final)
1	KH_2PO_4	27.2180	10	272.180	2.0000
	H_3BO_3	0.6183		6.183	0.1000
	$Na_2MoO_4 \cdot 2H_2O$	0.0242		0.242	0.0010
2	$CaCl_2 \cdot 2H_2O$	44.1060	10	441.060	3.0000
	KI	0.0830		0.830	0.0050
	$CoCl_2 \cdot 6H_2O$	0.0024		0.024	0.0001
3	$MgSO_4 \cdot 7H_2O$	49.3000	10	493.000	2.0000
	$MnSO_4 \cdot H_2O$	1.6902		16.902	0.1000
	$ZnSO_4 \cdot 7H_2O$	0.8627		8.627	0.0300
	$CuSO_4 \cdot 5H_2O$	0.0025		0.025	0.0001
4[b]	KNO_3	505.5500[b]	20	5055.500	50.0000
5[c]	$FeSO_4 \cdot 7H_2O$	0.8341	10	8.341	0.0300
	Na_2EDTA	1.1167		11.167	0.0300
6[d]	Nicotinic acid	0.0492	10	0.492	0.0040
	Pyridoxine · HCl	0.0822		0.822	0.0040
	Thiamine · HCl	0.1349		1.349	0.0040
7[d]	Myo-inositol	18.0160	10	180.160	1.0000
—	D-Glucose	—	—	21620.000	120.0000

[a] pH adjusted to 5.0 prior to autoclaving.
[b] Amount/2 l stock (g).
[c] Amber bottle.
[d] Refrigerate.

1. For maximum fiber production from fertilized ovules, use 0.5–5.0 μM GA_3. For occasional slight stimulation, also employ 5.0 μM IAA.
2. For maximum fiber production from unfertilized ovules, employ 5.0 μM IAA and 0.5 μM GA_3. For occasional slight stimulation, also employ 0.05 μM Kin and/or reduce KNO_3 to 45 mM and add 2–5 mM NH_4NO_3.
3. Two methods for induction for callus from cultured ovules (unfertilized) are (1) substitute fructose for glucose and employ 5.0 μM GA_3, or (2) use plastic culture vessels, delete boron, employ NH_4NO_3 as in # 3 above, and substitute sucrose for glucose.

been modified (for much of the subsequent work) to utilize only glucose. The basal medium, plus certain alterations and additions are listed in Table 1.

Epidermal cells, which become commercial lint fibers, initiate elongation at anthesis. It was concluded from in vitro experiments that fibers on isolated ovules continue to develop, in culture, only if fertilization was accomplished before harvest of the ovaries and transfer of ovules (BEASLEY, 1971). Continuing studies with fertilized ovules collected 48 h postanthesis (fertilization assured), showed that GA_3 induced a marked stimulation, and Kin and ABA a marked inhibition, of fiber development. IAA stimulated fiber production only slightly, if at all (BEASLEY and TING, 1973).

Using ovules collected from plants on the second day postanthesis (after fertilization was prevented by removing stigmas and stamens on the morning of

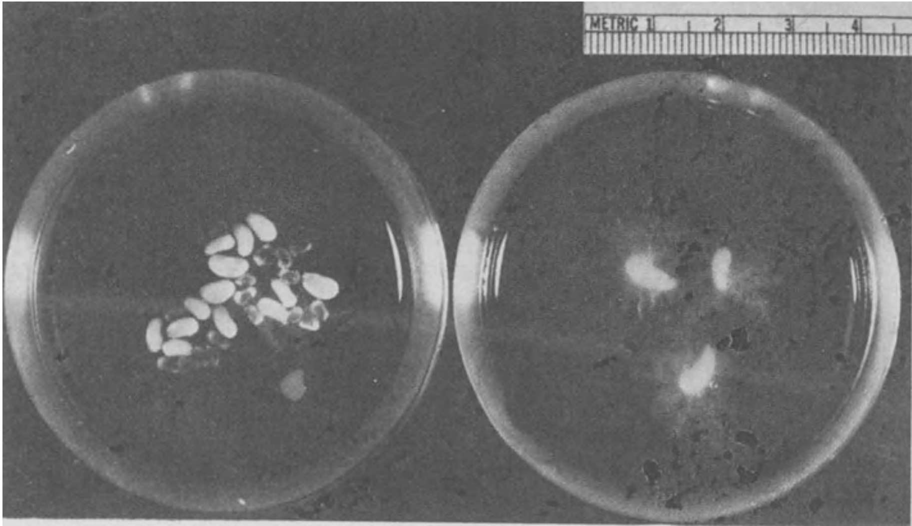

Fig. 1. White-fiberless and brown-shrunken ovules, all from a single ovary cultured in basal medium *(left)*; ovules with associated fibers cultured in basal medium containing 5.0 μM IAA and 0.5 μM GA_3 *(right)*

anthesis), it was shown that unfertilized ovules enlarge in the presence of Kin and both enlarge and produce fibers in the presence of IAA and/or GA_3. IAA provided for greater fiber production than did GA_3; combinations of the two hormones yielded approximately additive amounts of fiber (BEASLEY, 1973). The general response of unfertilized ovules to basal medium only, or basal medium containing IAA and GA_3 is shown in Figure 1. Both ABA and high Kin concentrations reduced the fiber production provided by IAA and GA_3. At low concentrations, Kin partially overcame the ABA inhibition of IAA - and GA_3-induced fiber development. High concentrations of combined GA_3 and ABA promoted callus formation from cultured ovules. Treatments decreasing total fiber production and increasing callus formation from cultured ovules cause, in turn, an increase in dry weight (BEASLEY and TING, 1974a). If no phytohormones were added to the culture medium containing unfertilized ovules, most of them browned, shriveled, and died (hereafter referred to as B-S ovules). However, the percentage of unfertilized ovules (no phytohormones added) that remained white and enlarged, but produced no fibers (hereafter referred to as W-F ovules), varied not only from carpel to carpel and boll to boll, but also with time of year that collection was made.

This same variability (in percentage of W-F or B-S ovules formed in the absence of exogenous phytohormones) was seen when similar unfertilized ovules were transferred to culture on the morning of anthesis. Using this modified collection procedure, it was shown that: (1) ABA increased the percentage of B-S ovules; (2) Kin increased the dry weight of nonfiber-producing, unfertilized ovules cultured in the presence of ABA; and, (3) these two hormones were generally antagonistic when applied in combination. Compared to results using basal

medium containing 50 mM KNO_3, inclusion of small amounts (2.5 mM) of NH_4NO_3 (maintaining total nitrogen the same) generally increased the percentage of W-F ovules. Greater amounts of NH_4NO_3 decreased the percentage of W-F ovules and the total dry weight from such unfertilized ovules cultured in the absence of phytohormones. In addition, it was established that increase in culture temperature from 28° to 34° C increased the percentage of W-F ovules recorded at the end of two weeks' culture in basal medium containing 50 mM KNO_3 as the sole nitrogen source. Irrespective of the disposition of control ovules (either W-F or B-S ovules at the end of two weeks culture in basal medium only), the addition of IAA and/or GA_3 provided for fiber development on unfertilized ovules collected on the morning of anthesis. It was confirmed that, in general, IAA and GA_3 provided for additive amounts of fiber production although slight synergism was occasionally noted. In addition, it was demonstrated that ovules acquire their capacity to respond to phytohormones between the third and second day preanthesis (BEASLEY and TING, 1974b). Ovules from flowers four days preanthesis and younger produced only callus and root-like appendages in response to IAA and GA_3.

Experiments with fibers isolated from ovules produced on the plant, showed that sugar nucleotide incorporation patterns vary with developmental age of the in vivo fibers, confirming that glucan biosynthesis differs qualitatively for primary and secondary wall formation (DELMER *et al.*, 1974). Those studies were expanded to include ovules and fibers cultured in vitro, and it was shown that similar (and even more rapid) patterns of sugar nucleotide incorporation into fibers existed for in vitro-cultured ovules (BEASLEY *et al.*, 1974b). That report also showed that unfertilized ovules need exposure to GA_3 for only a relatively short time (24 h) before transfer to IAA, in order for them to produce equal (or nearly equal) amounts of fiber as did ovules cultured in the continual presence of both hormones. As the length of GA_3 preculture increased from 24–48–72 h, prior to subtransfer of ovules to basal medium containing only IAA, the amount of fiber produced by the end of the culture period decreased.

Research efforts were continued not only to improve the basal medium, but also to determine which micronutrients might be most amenable to study via the cotton ovule procedure. Because of the apparent absolute requirement for exogenous IAA and/or GA_3 (no fibers produced in their absence) unfertilized ovules placed in culture on the morning of anthesis were the experimental material of choice for all subsequent studies. Experiments were conducted wherein calcium, manganese, magnesium, iron, or boron were deleted from a basal medium containing those hormones (IAA and GA_3) essential to fiber production from unfertilized ovules (BEASLEY *et al.*, 1974b). Maximum effort along these lines was expended on studies of the role of boron in ovule and fiber development. Deletion of boron not only reduced fiber production significantly but promoted the formation of extensive callus from the ovules. This callus production was shown to be due to the presence of GA_3 and not of IAA. In the presence of IAA, fiber development was limited by the absence of boron. In a boron deficient medium, IAA reduced the extent of callus formation due to GA_3 (BIRNBAUM *et al.*, 1974).

Major emphasis has been placed upon a review of literature pertinent to cotton ovule culture. For the interested reader, more extensive reviews and re-

ports on ovary and ovule culture with other species are available (JOHRI, 1962; KANTA *et al.*, 1962; MAHESHWARI and RANGASWAMY, 1963; PURI, 1963; JOHRI and SEHGAL, 1963; NITSCH, 1963; RAO, 1965; BAJAJ, 1966). Although numerous recent symposia have been held, and books and review articles published, on various aspects of plant tissue and cell culture, the review by BAJAJ and BOPP (1971) is the most recent that includes extensive citations on ovule and ovary culture.

5. Observations Useful in Expanding Research Utility

Although basic procedures have been established and major responses of in vitro-cultured ovules determined, it has repeatedly been stated (in our literature already cited) that environmental conditions influencing parent plants alter responses of ovules cultured in vitro. Similarly, it is recognized that a number of experimental modifications (e.g. sequential application of phytohormones, time of harvest, medium alterations) could produce information helpful in reducing laboratory variability of growth responses and/or provide for new concepts relative to ways of favorably manipulating field-grown cotton. This section presents a number of observations made during this project's development. It is not likely that these observations will, of themselves, contribute meaningfully to a deep understanding of the phenomena under investigation. It is hoped, however, that they may stimulate the interest of others working in similar areas and may thus provide some insight for more sophisticated experiments offering more detailed information.

5.1 Sequential Hormone Application

We reported (BEASLEY *et al.*, 1974b) that short term exposure (24 h) of day-of-anthesis ovules to basal medium plus GA_3 (0.5 μM), followed by transfer to basal medium containing IAA (5.0 μM) yielded the same or nearly the same (varying from 80–100%) extent of fiber as continual exposure of the ovules to both hormones. Converse treatments employing IAA as the preculture hormone, before transferring ovules to GA_3-containing medium, yielded no more fiber than treatments employing continual GA_3 exposure. We also reported on experiments showing that ovules lost their capacity to respond to GA_3 preculture as the time before exposure to IAA was increased. Since that report, we have determined that increasing the GA_3 concentration from 0.5 to 5.0 μM, during preculture periods, increases the subsequent response to continued IAA exposure, and have confirmed that ovules must also be exposed to IAA early in the culture period in order for them to produce maximum fiber (Fig. 2). Similarly, we established that increasing the preculture exposure time in basal medium, prior to transferring ovules to basal medium plus IAA, markedly decreases the fiber produced in response to IAA (Fig. 3). Even if the hormone treatment (following increasing time of preculture in basal medium only) consisted of both IAA and GA_3, the ovule's

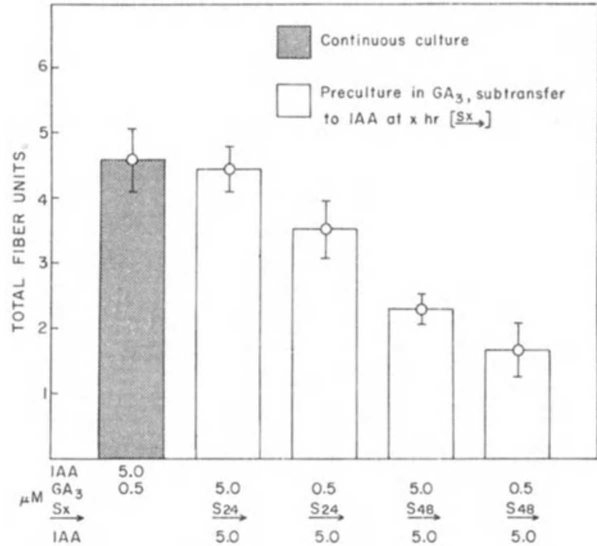

Fig. 2. Total fiber units at the end of two weeks from ovules initially transferred to culture on the morning of anthesis. Basal medium contained 5.0 µM IAA and 0.5 µM GA_3 for the continuous-culture treatment (no subtransfer). Other treatments contained basal medium plus 5.0 or 0.5 µM GA_3 for 24 h, or 5.0 or 0.5 µM GA_3 for 48 h, prior to subtransfer [S(x)] to basal medium plus 5.0 µM IAA. Averages obtained from 8 flasks (160 ovules) per treatment. Vertical bars indicate two standard error values above and two below the means

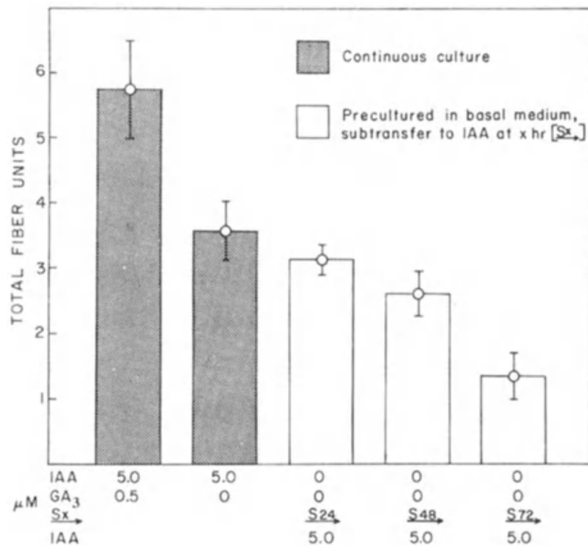

Fig. 3. Total fiber units at the end of two weeks from ovules initially transferred to culture on the morning of anthesis. Basal medium contained 5.0 µM IAA and 0.5 µM GA_3, or 5.0 µM IAA, for the continuous culture treatments (no subtransfer). Other treatments contained only basal medium for the first 24, 48, or 72 h prior to subtransfer [S(x)] to basal medium plus 5.0 µM IAA. Averages (data from two identical experiments) obtained from 11 flasks (220 ovules) per treatment. Vertical bars indicate two standard error values above and two below the means

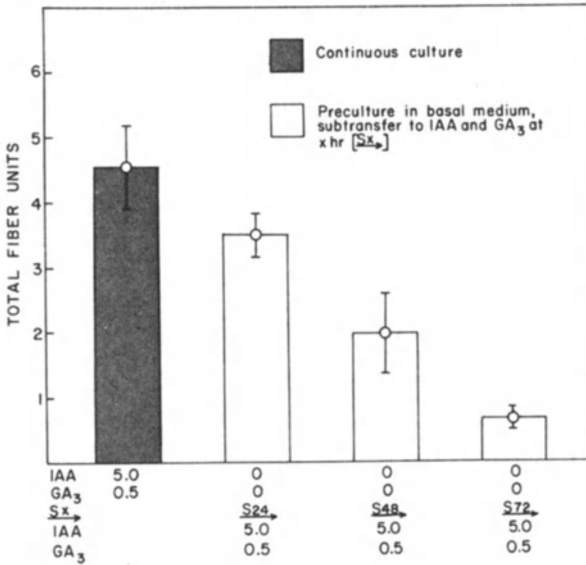

Fig. 4. Total fiber units at the end of two weeks from ovules initially transferred to culture on the morning of anthesis. Basal medium contained 5.0 μM IAA and 0.5 μM GA_3 for the continuous-culture treatment (no subtransfer). Other treatments contained only basal medium for the first 24, 48, or 72 h prior to subtransfer [S(x)] to basal medium plus 5.0 μM IAA and 0.5 μM GA_3. Averages obtained from 6 flasks (120 ovules) per treatment. Vertical bars indicate two standard error values above and two below the means

capacity to produce fiber was markedly decreased with increasing age postanthesis (Fig. 4). Despite the obvious significant differences between treatments and the generally repeatable GA_3 preculture effect, we occasionally noted that the usual preculture of ovules for 24 h in basal medium plus 0.5 μM GA yielded much less fiber (ca. 40%) than ovules cultured continuously in GA_3 and IAA. In such cases it was determined that longer periods of preculture with GA_3, before transfer to IAA, were ineffective in reestablishing maximum fiber production; but, increasing preculture periods in both IAA and GA_3, prior to transfer to IAA, increased the amount of fiber produced (Fig. 5). Thus, despite the apparent research potential for studying GA_3 preculture effects and the additivity of GA_3 and IAA responses (also adequately demonstrated with other plant research systems: GALSTON and MCCUNE, 1961; YANAGISHIMA, 1965; KAZAMA and KATSUMI, 1974), it is recognized that much remains to be done. It is suggested that the disposition of ovules to these hormones is likely modified, by still another substance(s), in response to altered environmental conditions surrounding the parent plants. As suggested before (BEASLEY and TING, 1973) ABA seems a prime candidate as the agent responsible for altering the fiber-promoting effects of auxins and gibberellins. It is further speculated that (1) the generally-agreed-upon differences between time of initiation for fuzz and lint fibers, and (2) the relatively distinct differences in length of the two fiber types, are due to sequential "perception" and relative amounts of effective endogenous auxins and gibberellins.

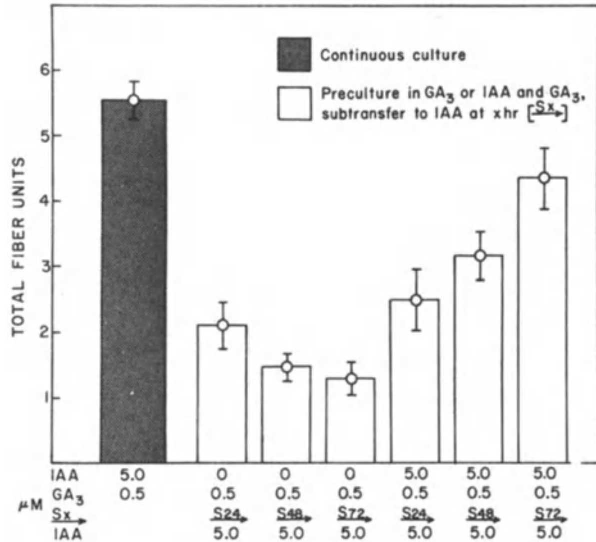

Fig. 5. Total fiber units at the end of two weeks from ovules initially transferred to culture on the morning of anthesis. Basal medium contained 5.0 μM IAA and 0.5 μM GA_3 for the continuous-culture treatment (no subtransfer). Other treatments contained 0.5 μM GA_3, or 5.0 μM IAA and 0.5 μM GA_3, for 24, 48, or 72 h prior to subtransfer [S(x)] to basal medium plus 5.0 μM IAA. Averages obtained from 8 flasks (160 ovules) per treatment. Vertical bars indicate two standard error values above and two below the means

5.2 Environmental Factors (Glasshouse) and Media Alterations (in vitro)

5.2.1 Nitrogen Source and Inositol

Several experiments have been conducted in which ovules were transferred to culture early in the morning, at noon, and late in the afternoon on the day of anthesis. Although much variability between experiments was noted, the trend was for ovules collected in the morning to produce the highest percentage of W-F ovules (see previous explanation of fiberless, growing or nongrowing ovules). Data from another unpublished experiment showed statistically significant decreases in percentage W-F ovules due to increasing concentrations of inositol (0, 0.125, 0.25, 0.5, 1.0 mM) in the basal medium containing 50 mM KNO_3. This trend of growth inhibition (due to inositol) of unfertilized ovules cultured in the absence of phytohormones was often seen; yet, variability of results prevented collection of consistently significant data.

Still another experiment added complexity without resolution of the factors governing the capacity of unfertilized ovules (without exogenous hormones) to grow in vitro. Ovules from day-of-anthesis flowers were harvested and transferred to basal medium containing 50 mM KNO_3 at 8:00 a.m., noon, and 4:00 p.m. In another treatment, the basal medium containing 50 mM KNO_3 was altered, for each time of collection, to contain 2.5 mM NH_4NO_3 and 45 mM KNO_3. Both of

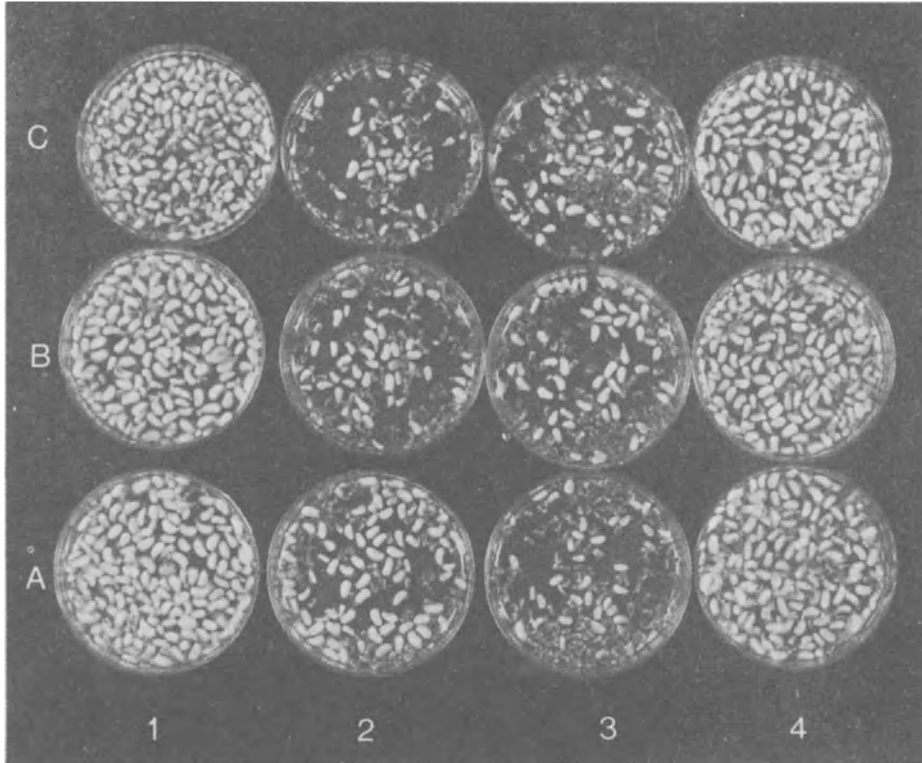

Fig. 6. White-fiberless and brown-shrunken ovules at the end of two weeks in various treatments. Flowers harvested and ovules transferred to culture at 8:00 a.m. C, noon B, 4:00 p.m. A, on the day of anthesis. Basal medium modified as follows: 1 45 mMKNO_3, 2.5 mM NH_4NO_3, plus 1.0 mM inositol; 2 same nitrogen as 1 but no inositol; 3 50 mM KNO_3 plus 1.0 mM inositol; 4 same nitrogen as 3 but no inositol

these treatments were split to contain no or the usual 1 mM inositol. Inositol significantly increased the percentage of W-F ovules when employed with NH_4NO_3 but decreased the percentage of W-F ovules when added to the basal medium using KNO_3 as the sole nitrogen source. In the former case, the percentage W-F tended to increase with time of harvest, but the converse was true when inositol was included with 50 mM KNO_3. This latter case confirmed the previous experiments indicating that more W-F ovules were recorded when obtained from ovaries collected early in the morning than from ovaries collected later in the day. For illustrative purposes, ovules pooled from replicated flasks of that experiment are shown in Figure 6.

The intentional nonfertilization of cultured ovules is an abnormal situation; however, the observed positive or negative response to such ovules (W-F or B-S) may provide clues to their environmentally triggered capacity to respond maximally to hormones generated as a result of the fertilization processes. The observation that Kin promotes the formation of W-F ovules, as do low concentrations of NH_4NO_3, seems related to information that (1) cytokinins and gibberellin

permit induction of nitrate reductase activity in the dark (LIPS and ROTH-BEJERANO, 1969) and (2) nitrate reductase activity varies seasonally and daily (ROTH-BEJERANO and LIPS, 1970; STEER, 1973). The synergistic induction of nitrate reductase by nitrate and benzylamino purine has also been reported (KNYPL, 1973). It is also of interest that inositol and NH_4NO_3 (KAUL and SABHARWAL, 1975) and inositol and adenine (quoted by MURASHIGE, 1974) interact in promoting growth and differentiation of plant tissue cultures.

5.2.2 Vitamins

Early in the establishment of a basal medium for the culture of fertilized cotton ovules, thiamine, nicotinic acid, and pyridoxine were added routinely even though a consistent requirement for them had not been established. Development of the unfertilized ovule culture method (fiber produced in response to exogenous IAA and GA_3) prompted reexamination of the requirements for vitamins. An experiment was conducted on ovules transferred to culture on the morning of anthesis; the basal medium for all treatments contained pyridoxine and nicotinic acid at 4.0 μM but thiamine was included at 4.0 μM (or excluded) for treatments containing 5.0 μM IAA, 0.5 μM GA_3, or combinations of these two hormones. Visual appearances suggested only slight or no decreases in fiber production due to the absence of thiamine. Total fiber units (as determined by the stain-destain method) and dry weights were recorded for all treatments, and average fiber lengths were determined for treatments containing IAA and GA_3. A highly significant reduction in TFU, due to deletion of thiamine, was recorded for the treatment containing both IAA and GA_3. For that treatment, the absence of thiamine reduced by 19%, 34%, and 48%, fiber length, dry weight, and TFU respectively. Data are shown in Figure 7. Similar experiments were conducted and for that particular treatment (IAA plus GA_3), reduction in fiber length varied from 12–20% in the absence of thiamine, with decreases in TFU varying from 40–52%. It appeared, thus, that the absence of thiamine (in the presence of pyridoxine and nicotinic acid) was not detrimental to fiber production supported by IAA, and was possibly involved in the additive nature of the IAA and GA_3 response. A possible explanation is that exogenous thiamine was not essential to continued elongation of lint fibers that had already initiated growth on the day of anthesis, but was essential for growth of integuments (including epidermal cell divisions) and development of new fiber initials beginning their elongation phase after the day of anthesis (after transfer to culture). In an effort to determine how the absence of thiamine would influence dose-response curves for IAA and GA_3, similar experiments with day-of-anthesis ovules were conducted, holding the level of IAA or GA_3 constant at their predetermined optima (5.0 and 0.5 μM respectively when applied alone) and varying the concentration of the other hormone (0.05, 0.5, and 5.0 μM). Both experiments were conducted in the presence and absence of thiamine, with the basal medium containing the usual amounts of pyridoxine and nicotinic acid. The absence of thiamine depressed total fiber production due to IAA or GA (in the presence of the optimum concentration of the other) yet the shape of the dose response curve was the same irrespective of the presence or absence of thiamine. Interpretation, relative to thiamine's primary involvement with either IAA or

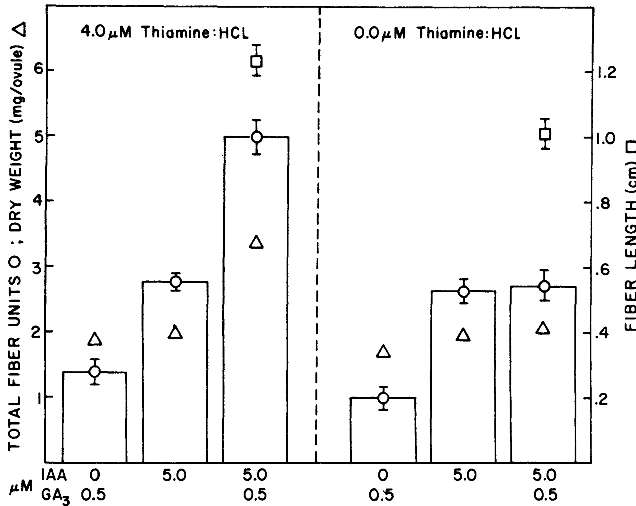

Fig. 7. Total fiber units, dry weight, and fiber length at the end of two weeks from ovules transferred to culture on the morning of anthesis. Basal medium contained 5.0 μM IAA, 0.5 μM GA_3, and 4.0 μM pyridoxine and 4.0 μM nicotinic acid. Thiamine was included at 4.0 μM or excluded. Averages (data from two identical experiments) were obtained from 12 flasks (240 ovules) per treatment. Vertical bars represent two standard error values above and two below the means

GA_3, remained elusive; however, it seemed that the presence of thiamine promoted GA_3-induced fiber production and ovule development more than it did IAA-induced growth.

5.2.3 Boron and Vitamins

Because of (1) the possible involvement of thiamine with new fiber formation (epidermal cell division and expansion of the integuments), and (2) the fact that extensive cell division (resulting in callus formation in vitro) originates within the integuments, it seemed logical to experiment with the presence or absence of thiamine in a culture situation known to promote callus formation. Such a situation could be created by culturing day-of-anthesis ovules in the presence of IAA and GA_3 and the absence of boric acid (BIRNBAUM *et al.*, 1974). All three vitamins together, or each employed singly, were used as treatments in the presence or absence of the normal amount of boric acid (0.1 mM). Fibers were produced in response to IAA and GA_3 in the presence of boric acid, and of the three vitamins, thiamine was responsible for maximum TFU. In treatments excluding boric acid, fiber production was essentially absent and heavy callus was formed; again, thiamine appeared to be the vitamin responsible for the growth (callus) increase (data from that experiment not included). Since previous research with boron had revealed that the presence of IAA reduced the extent of GA_3-induced callus in a boron deficient medium, we repeated the vitamin experiments with these two hormones added singly in a boron-deficient medium. It was conclusively demonstrated that thiamine was the vitamin essential for GA_3-induced callus formation when unfertilized ovules were cultured in the absence of boron (Fig. 8).

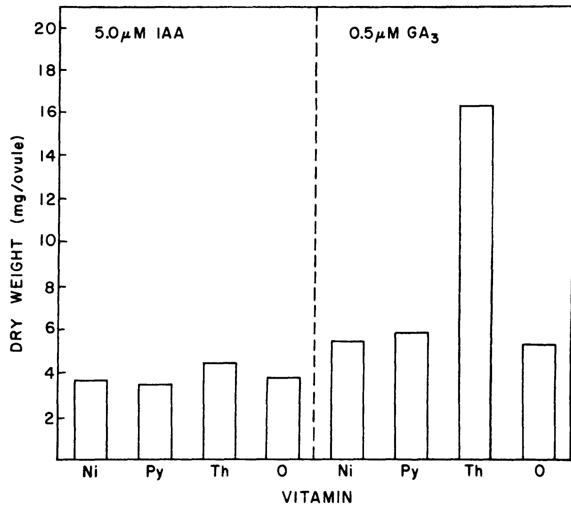

Fig. 8. Dry weight at the end of two weeks, in a boron deficient medium containing 5.0 μM IAA or 0.5 μM GA_3, from ovules transferred to culture on the morning of anthesis. When included, vitamins (Ni nicotinic acid, Py pyridoxine, Th thiamine) were employed at 4.0 μM. Averages obtained from 6 flasks (120 ovules) per treatment

Thiamine has clearly been demonstrated as the most critical vitamin in plant tissue culture research (MURASHIGE, 1974), and this seems particularly true for callus cultures. Results of experiments on roots have shown (SALISBURY and ROSS, 1969) that any two of the three vitamins under discussion provide for slight growth, and all three together provide for many-fold growth increases. These types of experiments (all possible combinations of the three vitamins) were also conducted with cotton ovules transferred to culture on the day of anthesis, but, marked synergism of the three (over any two) of the vitamins, was absent: thiamine remained as the critically-important vitamin for total fiber production. Since thiamine is furnished by leaves and will not translocate past a stem or petiole girdle, it is suspected that endogenous levels of this vitamin on the day of ovule transfer are adequate to maintain normal growth of initiating fibers; however, unless thiamine is added exogenously, development of those fibers subsequently initiating growth is severely reduced. It seems unlikely that a thiamine deficiency alone would be the cause of altered ratios of lint to fuzz fibers seen among cotton varieties. However, it is possible that more sophisticated experiments with thiamine might lead to information valuable in explaining the physiological and biochemical basis for altered lint-fuzz percentages.

6. Future Potential

The four major components of cotton yield are (1) plants/acre, (2) bolls/plant, (3) seed/boll, and (4) fiber-seed. Promising field research on the agronomics and genetics of narrow-row, high-density cotton should increase the ratio of the first

component and decrease the ratio of the second. From these alterations, growers should achieve an increase in bolls/acre, with bolls being set over a short period of time. Such an achievement will improve the potential for obtaining practical increases in seed and fiber via application of plant growth regulators. Continuing laboratory research with in vitro cotton ovules (and other components of the flower) will hopefully contribute to that end via increased understanding of developmental seed physiology and the hormonal mechanisms regulating fiber production.

This author holds the generalized concepts that (1) cotton bolls are retained on the plant only if adequate numbers of ovules have been fertilized and an optimum, endogenous hormone balance is maintained to prevent abortion, and (2) inadequate fertilization may result from failure of anthers to dehisce, from nonviable pollen, and possibly from nonreceptive ovules. For this discussion, ovules might be nonreceptive due to (1) incompatibility at the level of the gamete (egg cell), (2) nonfunctional cells or tissue formed from other haploid nuclei of the mature embryo sac, and/or (3) metabolic interference with the processes of auxin synthesis or transport to tissue external to the embryo sac.

Although it is not clear at this time, it is suspected that responses (W-F vs B-S ovules) of in vitro ovules (to a basal medium containing no exogenous hormones) are indicative of endogenous hormone balances, or imbalances, as altered by environmental conditions. For the interested reader, a brief (but succinct) review of environmental effects on seed development is recommended (NOGGLE, 1974). We have offered information relative to experimental variables that shift the in vitro ratio of growing to nongrowing unfertilized ovules. We also have information suggesting that certain environmental treatments of excised preanthesis floral branches alter the capacity of unfertilized ovules to produce fibers in response to exogenous IAA (unpublished and not mentioned previously). It now remains to delimit those environmental variables impinging upon parent plants that alter in vitro responses of cultured ovules. We may then seek answers to questions such as:

1. Given adequate pollen, how do night and/or day temperatures, light intensity and/or quality, and water and/or nitrogen status alter ovules' capacity to develop normally and thus provide for maximum boll retention?

2. Given a stress condition such as excessive temperature and reduced water availability for several days preanthesis, is it possible to obviate ovule death by whole plant sprays of certain growth substances?

Experiments along these lines should permit the establishment of precise procedures making possible a detailed investigation of the requirement for gibberellin promotion of maximum auxin response. Additionally, it is now possible to expand numerous studies on the relationships between plant hormones and carbohydrate source or nutritional elements. For example, we have concluded preliminary studies (to be reported elsewhere) that have investigated, in fibers of varying age, the roles of nutritional potassium and cellular malate as influenced by exogenous ABA. Those investigations on the processes of turgor could be expanded to include the interaction effects of ABA and its antagonists (e.g., auxins) on osmoregulation of cell elongation.

With respect to cell wall biogenesis, certain plant hormones have been shown to be intimately involved in those processes leading to primary wall formation. In

particular, auxins have been implicated as those hormones involved in glucan assemblage or turn over (ABDUL-BAKI and RAY, 1971). Even more specific is evidence that indoleacetic acid, ester-linked with various carbohydrates, results directly or indirectly in changes in physical and possibly chemical properties of the primary wall (BANDURSKI and PISKORNIK, 1973). Other workers have shown xyloglucan connection between cellulose fibers and pectic polymers in the primary wall (ALBERSHEIM *et al.*, 1973) and an indoleacetic acid-induced conversion of wall xyloglucan from insoluble to water-soluble form (LABAVITCH and RAY, 1974).

It is important to mention that cottons which produce only lint fibers (so-called naked seed) respond in vitro to GA_3 and IAA in a manner identical to Acala SJ-1, a cotton producing both lint and fuzz (unpublished). Employing such naked-seed varieties for in vitro studies should remove complications (in interpretation of results) imposed by the presence of fuzz fibers (see BEASLEY *et al.*, 1974b).

The potential for improving seed set and development through hormone application under adverse environmental conditions has already been mentioned. A greater potential may exist, however, in employing the technique of ovule culture as an aid to practical plant breeding. Visual identification of superior individual plants is the first step in selection procedures of a breeding program, however, it is likely that numerous subtle but significant differences between plants exist that cannot be detected by current progeny-row procedures. We have presented evidence that hormone balances (or imbalances) govern the capacity of unfertilized cotton seed to grow and produce fiber in vitro. We have speculated that alterations in the environment surrounding parent plants cause shifts in endogenous hormone levels and, thus in the resultant capacity of unfertilized ovules to grow in vitro. We have also observed that all such ovules within a single ovary may grow in vitro (W-F ovules), or that all ovules from another ovary (from a different plant, but grown under identical environmental conditions) may die in vitro (B-S ovules). It seems possible, then, to determine if individual plants consistently produce a preponderance of one or the other of these types of unfertilized ovules. If it is demonstrated that such is the case, and that adverse environmental conditions are correlated with the ratio of W-F to B-S ovules in vitro, it may be possible to select plants resistant to those conditions (e.g., water and/or temperature stress) which reduce seed set and fiber development.

In addition to being a selection tool for plant breeders, ovule culture methods offer potential for providing homozygous pure lines to be used as commercial varieties or as parents for the production of hybrid or synthetic varieties. The need for large numbers of doubled haploids has fostered research leading to the production of androgenic, monoploid plantlets (see Chap. II.1 of this Vol.) from several economically valuable plant species. No means have been developed for the in vitro formation of monoploids from cotton, nor have monoploid plants from any species been derived in vitro from megagametes cultured in vitro to the point of germination within the unpollinated ovule. Since the encasement for the zygote (and for a potential megagametic monoploid) has been grown in vitro, and since normal development of the nucellus, integuments, and epidermis (fiber cells) of the ovule has been achieved through application of exogenous hormones, it now remains to undertake those studies aimed at induction of division in the mature, unfertilized egg cells, or haploid nuclei of the preanthesis embryo sac.

Cotton ovule culture has already been used to improve the success of making a hybrid cross (PUNDIR, 1974).

7. Summary

Most fundamental research methods in biology are developed and accepted widely because the system selected for study offers unique characteristics or advantages with respect to ease of manipulation, genetic composition, rapidity of results, freedom from artifacts, and the like. Although cotton ovule culture does not satisfy all of these criteria for all laboratories, it offers certain advantages not afforded by any other research method known to this author. Foremost is the advantage that in unfertilized ovules, growth of their associated fibers is under strict control by gibberellin and/or auxin. Without at least one of these, no fiber growth is obtained. Furthermore, both primary and secondary wall formation (final maturation) are achieved in vitro in the synchronously-elongating fiber cells, without complications imposed by cell division and/or callus formation. Although the following characteristics are not unique, the culture system employs a completely defined medium, permits utilization of a large sample size (obtained aseptically with speed and ease), and allows exposure to, or removal from, various exogenous plant hormones at any stage of fiber-cell development.

Both fertilized and unfertilized cotton ovules have been cultured in vitro. Ovules and their associated fibers respond in vitro to four main classes of hormones: auxins, gibberellins, cytokinins, and abscisic acid. Interactions of these hormones have been described. The in vitro methodology lends itself to detailed investigations of nutritional requirements for both seed and fiber development. In addition to exogenous hormones, minor constituents of the basal culture media (e.g., vitamins and inositol) seem to be involved in cellular processes which are of interest for both fundamental and practical reasons.

The variability in responses of unfertilized cotton ovules in vitro has plagued the development of research methods. However, it is suggested that perhaps the variability in ovule response in vitro (from boll to boll, with season of the year, and with time of day) can be used to advantage in selection of superior plants for breeding purposes. Furthermore, the general methodology may be followed in future attempts to create isogenic lines useful in a cotton breeding program.

It is concluded that a new research tool is available, not only for the cotton industry, but for studies in plant physiology that seek to answer the many unknown questions concerning mechanisms of hormone action, cell wall biogenesis, and seed and embryo development.

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References see page 207.

10. Regeneration of Plants from Tissue Cultures

S. NARAYANASWAMY

1. Introduction

In recent years, with success in the culture of isolated plant parts as callus, and growth of free cells and cell groups as suspensions by the pioneering efforts of WHITE, GAUTHERET, HILDEBRANDT, REINERT, TULECKE, NICKELL and others in the 1950s, studies on the physiology of growth and differentiation can be said to have developed on a broader front. Organ redifferentiation can be manipulated in dedifferentiated tissue (callus) by subjecting it to the interacting influence of a whole host of plant hormones and nutrient constituents.

Many tissues of diverse origin from plants belonging to different taxonomic groups have been established in culture and the factors controlling growth and differentiation in the several tissues ascertained (see BAJAJ and BOPP, 1971; VASIL and VASIL, 1972; MURASHIGE, 1974). The subtle interactions among the different groups of plant hormones, auxins, cytokinins, gibberellins, abscisin and ethylene—operative either directly or indirectly, alone or in synergistic combinations have given us an insight into the interrelationships existing between cells, tissues and organs, and in the integrated development of a whole plant. Regeneration has been induced in many sorts of calli but one cannot do so every time on any callus.

This article reviews the various aspects concerned with culture methods, nutritional requirements of the cells and tissues for growth and morphogenesis, factors affecting regeneration etc., in the light of recent advances in the subject. The prospects of the use of cell and tissue culture techniques as an alternative method to the mechanism of sex in large-scale propagation of plants of economic and horticultural importance, will also be elucidated.

2. Induction of Callus

Multicellular systems are highly organized. Cells constituting a tissue and different tissue systems operate in a highly coordinated manner. An organized tissue such as a root or a stem, can be completely changed to a rapidly proliferating undifferentiated mass of cells (callus), if cultured on a nutrient medium containing specific growth hormones such as IAA[1], NAA, IBA, BTOA, 2,4-D, Kinetin and

[1] Abbreviations used: IAA (indole-3-acetic acid): NAA (naphthaleneacetic acid): IBA (indole-3-butyric acid): BTOA (2-benz-thiazolylacetic acid): 2,4-D (2,4-dichlorophenoxyacetic acid): GA_3 (gibberellic acid): 2 iP (N_6-(2-isopentenyl-adenine): YE (yeast extract): ME (malt extract): ABA (Abscisic acid): BAP (6-benzylaminopurine): CH (casein hydrolysate).

other purine derivatives. The combination of a cytokinin with an auxin strongly enhances callus induction. Reports of the effect of GA_3 in promoting callus growth in general are conflicting (SCHROEDER and SPECTOR, 1957; BERGMANN, 1958; NÉTIEN, 1958; NICKELL and TULECKE, 1959) although in some instances stimulation of cambial activity in stem segments and cell division in free cell suspensions (DIGBY and WAREING, 1966a, b) have been observed. A spurt in growth of both callus and shoot buds in tobacco tissue occurred with increasing levels of both GA_3 and 2 iP (ENGELKE *et al.*, 1973) via culture medium. Monocotyledonous tissues were inhibited by GA_3 without exception (BUTENKO, 1968).

Excised plant parts are cultured in any one of the standard media and incubated under controlled conditions of temperature, light and humidity. This represents "organ culture" as a prelude towards initiation of a true plant tissue culture in the form of a callus. The cells are stimulated to undergo dedifferentiation to give a proliferated mass of tissue, the cultured organ losing its specialized structure in 3–4 weeks of incubation. When small pieces of tissue are transferred to fresh media of similar or different composition under aseptic conditions, further growth occurs forming a typical callus, freed from the influence of the parent explant. The callus can be maintained for an unlimited period by subculture i.e., transfer to fresh nutrient medium every four weeks for use in different experiments. A tissue culture thus established can be grown in liquid media under agitation as suspension culture. Mesophyll cells from leaves could be isolated by gentle maceration and the filtered cell suspensions used for obtaining cell colonies and regeneration of plants from mesophyll protoplasts (see Chap. IV.1 of this Vol.).

3. Composition of Culture Media

The composition of the culture medium is an important factor in the successful establishment of a tissue culture. Culture conditions favoring callus growth may not be suitable for organ differentiation. Each tissue type requires a different formulation, depending on whether the objective is to obtain optimum growth rate or induce organogenesis.

Several media have been developed by various workers to suit particular requirements of a cultured tissue. A standard or basal medium consists of a balanced mixture of macronutrient and micronutrient elements (salts of chlorides, nitrates, sulphates, phosphates and iodides of Ca, Mg, K, Na, Fe, Mn, Zn, and boron), vitamins, a carbon source, organic growth factors (aminoacids, urea and peptones), a source of reduced nitrogen supply and plant hormones.

Addition of deproteinized coconut milk, tomato juice, water melon juice, orange juice and other plant extracts, YE, ME or protein hydrolysate brings about mitosis in quiescent cells and has proved beneficial, though not essential, in cases where the tissues are otherwise recalcitrant on a purely synthetic medium. Divergent nutrient media have been used and they are summarized in Table 1(a–c).

The media of GAMBORG *et al.* (1968) and SCHENK and HILDEBRANDT (1972) have been formulated to support the growth of a wide variety of tissues of both

Table 1a. Major inorganic salts present in various nutrient media (all values expressed as mg/l)

Constituent	GAUTHERET (1942)	HILDEBRANDT *et al.* (1946)	BURKHOLDER and NICKELL (1949)	NITSCH (1951)	HELLER (1953)	REINERT and WHITE (1956)	MURASHIGE and SKOOG (1962)	WHITE (1963)	GAMBORG *et al.* (1968)	SCHENK and HILDEBRANDT (1972)
$(NH_4)_2SO_4$	—	—	—	—	—	—	—	—	134	—
$MgSO_4 \cdot 7H_2O$	125	180	246	250	250	360	370	720	500	400
Na_2SO_4	—	800	—	—	—	200	—	200	—	—
$MgCl_2 \cdot 6H_2O$	—	—	203	—	—	—	—	—	—	—
KCl	—	65	149	1500	750	65	—	65	—	—
$CaCl_2 \cdot 2H_2O$	—	—	441	25	75	—	440	—	150	200
$NaNO_3$	—	—	—	—	600	—	—	—	—	—
KNO_3	125	80	202	2000	—	80	1900	80	3000	2500
$Ca(NO_3)_2 \cdot 4H_2O$	500	400	708	—	—	200	—	300	—	—
NH_4NO_3	—	—	—	—	—	—	1650	—	—	—
$NaH_2PO_4 \cdot H_2O$	—	33	—	250	125	16.5	—	16.5	150	—
$NH_4H_2PO_4$	—	—	—	—	—	—	—	—	—	300
KH_2PO_4	125	—	1088	—	—	—	170	—	—	—

Table 1b. Inorganic micronutrients present in various media (expressed as mg/l)

Constituents	GAUTHERET (1942)	HILDEBRANDT et al. (1946)	NITSCH (1951)	HELLER (1953)	REINERT and WHITE (1956)	MURASHIGE and SKOOG (1962)	WHITE (1963)	GAMBORG et al. (1968)	SCHENK and HILDEBRANDT (1972)
$NiSO_4$	0.05	—	—	—	—	—	—	—	—
$FeSO_4 \cdot 7H_2O$	—	—	—	—	—	27.85	—	—	15
Na_2EDTA	—	—	—	—	—	37.25	—	—	20
$MnSO_4 \cdot 4H_2O$	3	4.5	3	0.1	4.5	22.3	7	10	10
$ZnSO_4 \cdot 7H_2O$	0.18	6	0.5	1	1.5	8.6	3	2	1.0
$CuSO_4 \cdot 5H_2O$	0.05	—	0.025	0.03	—	0.025	—	0.025	0.2
$BeSO_4$	0.1	—	—	—	—	—	—	—	—
H_2SO_4	1	—	0.5	—	—	—	—	—	—
$Fe_2(SO_4)_3$	50	—	—	—	2.5	—	2.5	—	—
$Ti(SO_4)_3$	0.2	—	—	—	—	—	—	—	—
$NiCl_2 \cdot 6H_2O$	—	—	—	0.03	—	—	—	—	—
$CoCl_2 \cdot 6H_2O$	0.05	—	—	—	—	—	—	0.025	0.1
$AlCl_3$	—	—	—	0.03	—	0.025	—	—	—
$FeCl_3 \cdot 6H_2O$	—	—	—	1	—	—	—	—	—
Ferric tartarate	—	40	—	—	—	—	—	—	—
$FeC_6O_5H_7 \cdot 5H_2O$	—	—	10	—	—	—	—	—	—
Fe (as Sequestrene)	—	—	—	—	—	—	—	28	—
KI	0.5	3	0.5	0.01	0.75	0.83	0.75	—	1.0
H_3BO_3	0.05	0.38	0.5	1	1.5	6.2	1.5	3	5
$Na_2MoO_4 \cdot 2H_2O$	—	—	0.025	—	—	0.025	—	0.25	0.1
$NH_4H_2 \cdot PO_4$	—	—	—	—	—	—	—	—	300

Table 1c. Organic constituents in nutrient media (expressed as mg/l)

Constituents	GAUTHERET (1942)	HILDEBRANDT *et al.* (1946)	NITSCH (1951)	HELLER (1953)	REINERT and WHITE (1956)	MURASHIGE and SKOOG (1962)	WHITE (1963)	GAMBORG *et al.* (1968)	SCHENK and HILDEBRANDT (1972)
Sucrose	30 $g l^{-1}$	20 $g l^{-1}$	50 $g l^{-1}$ or 36 $g l^{-1}$	20	—	30—50 $g l^{-1}$	20 $g l^{-1}$	20 $g l^{-1}$	30 $g l^{-1}$
Glucose	—	—		—	—	—	—	—	—
Myo-inositol	—	—	—	—	100	100		100	1000
Nicotinic acid	0.5	—	—	—	0.5	0.5	0.5	1	0.5
Pyridoxine HCl	0.1	—	—	—	0.1	0.5	0.1	—	0.5
Thiamine HCl	0.1—1	0.1	1	1	0.1	0.1	0.1	10	5
Calcium pantothenate	—	—	—	—	0.1	—	1	—	—
Biotin	—	—	—	—	0.01	—	—	—	—
Glycine	3	3	—	—	—	2	3	—	—
Cystenic HCl	10	—	10	—	3	—	1	—	—

monocotyledons and dicotyledons and perhaps even of conifers. WOLTER and SKOOG (1966) used a synthetic medium modified after REINERT and WHITE (1956) for growth of *Fraxinus* tissue cultures. LINSMAIER and SKOOG (1965) medium is essentially similar to MURASHIGE and SKOOG's (1962) except in the content of organic constituents.

A basal medium that initiates callus growth may not be suitable for maintaining its growth or for organ induction. In general, callus growth cannot be sustained unless the medium is fortified by glycine or L-glutamic+L-aspartic acids and their amides, L-arginine or L-tyrosine or a mixture of 18 amino acids as in REINERT and WHITE (1956) and a complex mixture of vitamins.

Iron is added in the form of ferric citrate or better as a chelate of EDTA (ferric-sodium-ethylenediamine-tetra acetate) in order to ensure its availability over a wide range of pH of the medium. Special organic ingredients are also used: riboflavin, folic acid, choline chloride, ascorbic acid, cyanocobalamin and p-aminobenzoic acid. Plant extracts and solutions that are thermo-labile are sterilized by millipore-filtration or through pyrex sintered glass before addition to the basal medium.

The pH of the nutrient solution which is a balanced mixture of macro- and microelements is adjusted to 5.6–6.0 by the addition of 0.1 HCl or NaOH. While "Difco" Noble bacteriological agar (0.6–1% w/v) is preferred in studies of nutrition and metabolism, other grades of agar could be used to gel the medium for routine purposes. The nutrient media with dissolved agar are dispensed into pyrex culture tubes or conical flasks and capped with polypropylene tube closures, Morton caps, or plugged with cotton. While callus can be maintained satisfactorily on nutrient agar (stationary culture), liquid media under agitation are also used. The media are autoclaved at 15 lb/psi for 15 to 20 min at 121° C. Standard procedures for the preparation of the various media are described by GAUTHERET (1959), WHITE (1963) and BUTENKO (1968). The cultures are kept for incubation at constant temperatures (25° C ± 2° C) and humidity under 16 h daily illumination provided by cool white fluorescent tubes of approximately 1000 lux or 20 W Gro lux lamps, and can be studied under variable conditions.

4. Aseptic Preparation of Plant Material for Culture

Seedlings that are 8–10 days old, raised from seeds germinated under aseptic conditions, provide a suitable source of surface sterile material for initiating callus cultures from root, stem, hypocotyl, shoot tip, and cotyledons than field-grown plants. The seeds are surface sterilized with a 10% (w/v) filtered solution of calcium hypochlorite from 20–30 min to a few hours, the duration depending upon the hardness of the seedcoat. A solution of $HgCl_2$ (0.1%) or bromine water (1–2% w/v) is also used in difficult cases but this needs thorough washing in sterile water. H_2O_2 (10–12%) is another widely used sterilizing agent. Stem and floral segments, pith, cortical, and vascular parenchyma, medullary ray parenchyma, epidermal and subepidermal tissue, vascular cambia of tree species, storage par-

enchyma of tubers, petals, root, and shoot tips, leaf mesophyll, cotyledons, bulb scales, ovules, ovaries, endosperm, anther parts—in fact any part of the plant can now be successfully brought into culture.

5. Callus: Growth Characteristics

Calli of different species may vary in their texture, friability and coloration. They may be pale yellow or albino, chlorophyllous or anthocyanin-pigmented. Stem tissue culture of *Citrus grandis* gave callus strains that were greenish-white, compact and nodular, but slow growing, or that were highly friable and fast growing (MITRA and CHATURVEDI, 1972). The pigmentation may be uniform or patchy, certain regions remaining unpigmented. Callus tissues of *Parthenocissus* (STANKO and BRADINSKAYA, 1962), *Haplopappus* (CONSTABEL *et al.*, 1971), *Dimorphotheca* (BALL *et al.*, 1972) and corn endosperm (STRAUS, 1959) are anthocyanin-producing. In general the pigmentation is the same as the tissue of origin of the callus and this may be lost by repeated subculture (IBRAHIM *et al.*, 1971). In stem tissue cultures of *Plumbago*, HEBLE *et al.* (1974) noted segregation of variant cell lines differing in growth potential, morphology and pigmentation. Anthocyanin-producing and nonproducing cell lines have been isolated from tissue cultures of carrot (ALFERMANN and REINHARD, 1971). Green-pigmented callus grew better under light than in the dark. Pigmentation is considerably influenced by the level of dextrose, presence of soluble starch, nitrogen deficiency, temperature, light and exogenous auxin.

Certain strains of callus that required auxin in the initial stages of growth acquired the capacity for continued growth by dedifferentiation without exogenous auxin, referred to as "anergy" by GAUTHERET (1955). *Nicotiana bigelovii* tissues could grow without hormones after initial culture on a 2,4-D medium (BUIATTI and BENNICI, 1970). Such "habituated" tissues may not differ from the normal tissues in cytology but, in general, were either incapable of organ induction or possessed only a low potential for morphogenesis (GAUTHERET, 1959; LUTZ, 1970).

A callus consisting wholly of uniform parenchyma cells is witnessed only rarely, such as in *Agave* and *Rosa*. It depends on the source and origin of callus, ingredients in the nutrient media and duration of subculture. The cells in a callus may consist initially of a variety of cell types, sizes and shapes differing in their vacuolation, cell contents and wall thickening. Lignified elements, tannin-bearing and secretory cells may be present as growth proceeds. Cytodifferentiation in the form of tracheids, sieve elements, parenchyma cells, cambia and periderm may develop during growth and maturation of the callus under chemical stimuli.

Individual cells or groups of cells of smaller dimensions may form small nests of tissue scattered throughout the callus, the so-called meristemoids which may form centres of further cell proliferation or become transformed into cyclic nodules from the perivascular regions of which shoot apices, root primordia or incipient embryos may differentiate (VASIL and HILDEBRANDT, 1966a, b). The

vascular nodules may be formed in the peripheral regions of the callus as in *Pelargonium* (CHEN and GALSTON, 1967) or endogenously as in most other tissues. Hormone and metabolite concentration controls the nature of organogenesis in the tissue. Differentiating vascular tissues may produce plant hormones and the physiological response of the tissue is altered, depending on the exogenous supply of growth factors interacting with the internal milieu.

Cytological changes may occur in somatic cells in vitro (KAO *et al.*, 1970; SHIMADA, 1971; HEINZ and MEE, 1971) leading to the development of polyploid and aneuploid cells as growth proceeds, so that the tissue becomes mixoploid although originally the cells may all be diploid. Diploid cells may become polyploids: through endoreduplication or selective stimulation to cell division of a polyploid cell already present (PARTANEN, 1963; D'AMATO, 1965, see Chap III.1 of this Vol.), and this may occur even quite regularly in cell cultures (MURASHIGE and NAKANO, 1965, 1967). Callus cultures maintained by subculture may consist solely of cells that are tetraploid or octoploid and even aneuploid, with fewer diploid cells or none at all, and are, therefore, distinguished by a considerable genetical inhomogeneity.

This condition may be induced by culture of explants characterized by polysom of cells as for example, the pith parenchyma of tobacco (SHIMADA and TABATA, 1967). *Citrus* tissue proved to be all tetraploid in the course of a year under culture (MURASHIGE *et al.*, 1967). On the other hand, certain other species have retained their diploid nature in cultured tissue (REINERT and KÜSTER, 1966; KAO *et al.*, 1970). It is also possible that prolonged subculture may lead to the establishment of the dominant karyotype in in vitro cell populations i.e., the diploid state through selection pressure (SINGH *et al.*, 1972).

An ideal tissue culture characterized by the possession of stable but fewer chromosomes, absence of endoploidy, capacity for exuberant growth on a chemically defined medium and photosynthetically autotrophic, easy friability, high plating efficiency and high morphogenetic potential, is however, yet to be achieved (NICKELL and TORREY, 1969).

6. Organogenesis in Callus Cultures

Organization can be brought about in callus by the controlled initiation of an organ primordium through manipulation of the nutrient and hormonal constituents in the culture media. Regeneration occurs readily in a simpler medium than is needed for a tissue culture. Normally the medium contains organic and inorganic elements, and exogenous phytohormones. Each tissue has its own requirements and sometimes less defined growth adjuvants play a part in organogenesis. The phenomenon is also dependent upon a number of other factors such as source of origin of the callus, its genotype and age, endogenous hormone levels and various physical factors.

That grafting of buds into the callus can bring about xylogenesis was demonstrated by WETMORE and SOROKIN as early as 1955. In the organization of a vascular nodule, the complex role of a sugar (in conjunction with an auxin and cytoki-

nin), particularly a disaccharide containing a-glucosyl radical at the nonreducing end and IAA, is implicated (ROBERTS, 1968). The type of vascular differentiation depended upon the concentration of sucrose in the medium. Xylogenetic effects could be induced by the addition of BAP and starch in cultured tuber tissue of *Helianthus tuberosus* whereas GA_3 or ABA inhibited tracheid differentiation (MINOCHA and HALPERIN, 1974). Direct differentation of a cell in isolation into a tracheid, sieve element or a sclereid in a defined or complex media, has however seldom been realized, though xylogenic stimuli have been observed in cells that have undergone at least one division (HALPERIN, 1973).

Species which readily regenerate in vivo may do so easier in vitro, though not necessarily so. Rooting occurs more often than other forms of regeneration, irrespective of the source of the callus and is sometimes too random to define its conditions. Shoot formation followed by rooting is characteristic of cultures that are freshly isolated from hypocotyl, stem and leaf segments, and rarely from root callus (with the exception of carrot), although isolated roots in culture have given rise to buds as in tomato (NORTON and BOLL, 1954), *Convolvulus* (TORREY, 1958) and *Brassica* (BAJAJ and NIETSCH, 1975). In *Atropa*, callus formed at the base of the tissue culture-induced roots regenerated a shoot bud (THOMAS and STREET, 1972). Endogenous growth regulators supplied by the vascular tissue may play an important role in bud initiation.

In most calli, shoot formation may decrease with age and subculture, but capacity for rooting may persist for years, as in sugarcane (BARBA and NICKELL, 1969). Initiation of shoot buds may precede rhizogenesis or vice versa, or the induced shoots may grow as rootless shoots as in *Pergularia* (PRABHUDESAI and NARAYANASWAMY, 1974) and many other plants.

The callus may remain undifferentiated though showing prolific growth, regardless of the hormones and metabolites to which they are exposed. Those showing exuberant growth are least conducive for regeneration, as observed in stem callus of *Holarrhaena* (HEBLE *et al.*, 1971) and *Citrus* (CHATURVEDI *et al.*, 1974a, b), and organ neoformation generally follows cessation of unlimited proliferation.

Most *Nicotiana* species are bud-forming types and show greater propensity for adventive bud formation than embryos in culture, and carrot tissues are more characterized by regeneration through embryos than shoot buds.

Since the elegant demonstration by SKOOG and MILLER (1957) that the relative ratio of cytokinin (kinetin) to auxin (IAA) determined the nature of organogenesis in tobacco pith tissue, quantitative changes have been found to be decisive in a number of other plant tissue systems. High concentrations of kinetin caused bud initiation, whereas high levels of auxin favored rooting. Zeatin (=2 iP) and (±)-dehydrozeatin—the two naturally occurring cytokinins isolated from *Zea mays* and *Lupinus luteus* respectively, proved equally effective in bud induction in tobacco (YAMADA *et al.*, 1972). The group attached to the N^6 position of cytokinins binds to a receptor site to bring about organ formation and in this reaction the levo-rotatory cytokinins caused shoot bud induction. Certain phenolic compounds enhanced shoot initiation in tobacco callus, probably due to the inactivation of auxin and a consequent rise in the physiologically effective level of cytoki-

nins (LEE and SKOOG, 1965). The molecular mechnism of action of the phytohormones is rather obscure.

N-1 naphthyl-thalmic acid which is an inhibitor of auxin also caused the induction of shoot buds (FENG and LINCK, 1970). In *Plumbago*, addition of adenine in the medium created conditions conducive for the differentiation of shoot buds which were all diploid, especially favoring the multiplication of diploid cells in a mixoploid callus (NITSCH *et al.*, 1967). An enhancement in embryogenic potential by 2,4,6-trichloro-phenoxy acetic acid—an auxin antagonist, via culture media, has been observed in tissue cultures of *Daucus carota* (NEWCOMB and WETHERELL, 1970). KOCHHAR *et al.* (1970) observed that the chelating agent, 1,3-diamino-2-hydroxypropane-N,N,N′,N′-tetraacetic acid brought about initiation of buds in haploid tobacco cultures. The same authors (1971) report that polycyclic hydrocarbons such as dibenz (a,h) anthracene, benz(a) pyrene, chrysene etc., can replace both auxin and cytokinins in shoot and root formation in haploid tobacco callus. MAJUMDAR and NEWTON (1972) have investigated the morphogenetic and cytogenetic effects of 7-12-dimethyl-benz-A-anthracene on *Haworthia* callus in vitro, but the morphogenetic action of these chemicals remains to be confirmed by studies on a variety of other tissues. Addition of ABA in place of cytokinin in the medium could provoke adventitious bud formation in root tuber tissue of sweet potato and stem tuber tissue of potato.

The role of exogenous nicotine as a factor in inducing morphogenesis in tissue cultures of *Nicotiana rustica* has been demonstrated by PETERS *et al.* (1974). Optimal nicotine concentration for root induction was 50 mg/l, although addition of nicotine was not necessary to induce differentiation in various other species of tobacco. Their occurrence in plant species that are easily amenable to morphogenesis would indicate a positive role for alkaloids in tissue differentiation.

Since differentiation is brought about by the synthesis of nucleic acids and proteins, many attempts have been made to correlate the two phenomena. VASSEUR (1972) has observed that shoot initiation in *Cichorium intybus* was associated with alterations in the pattern of RNA synthesis and nucleotides. An increase in the ratio of RNA/DNA and histone/DNA was related to organogenesis in tobacco, and to embryogenesis in carrot with DNA synthesis. A correlation of shoot formation with starch accumulation and the rate of respiration of the shoot-forming tissue has been reported (THORPE and MEIER, 1972).

Regenerated plantlets from callus cultures may show genetic variability and deviate from the normal diploids in being polyploids, which is reflected in various kinds of morphological abnormalities.

Organogenesis is generally dependent upon the size of the explant cultured. The smaller the explant, the less has been the regenerative ability, the larger ones consisting of parenchyma, vascular tissue and cambium showing spontaneous initiation of shoot buds irrespective of the auxin-cytokinin concentration (OKAZAWA *et al.*, 1967). However, small groups of homogeneous tissue taken from the epidermal and subepidermal layers of tobacco, used as experimental systems, could directly give rise to complex organs such as flower/buds/roots (TRAN THANH VAN, 1973). This could also be achieved from culture of relatively small explants of *Nautilocalyx* composed of only the superficial tissue freed from the correlative influences of other tissues (TRAN THANH VAN and DRIRA, 1970).

The source of the explant cultured is important in determining the regenerative potential. Leaves and leaf fragments of many plant species have shown capacity to form adventitious buds: *Begonia* (SHIGEMATSU and MATSUBARA, 1972), *Saintpaulia* (RAO and MOREL, 1974), *Crepis capillaris* (YONEDA, 1969), *Echeveria elegans* (RAJU and MANN, 1971), *Helionopsis orientalis* (KATO and KAWAHARA, 1972), members of the Solanaceae (GUPTA *et al.*, 1966; ZENKTELER, 1972; PRABHUDESAI and NARAYANASWAMY, 1973), cowpea (INDIRA and RAMADASAN, 1967); root sections of *Convolvulus* (BONNETT and TORREY, 1965); *Ipomea batatas* (GUNCKEL *et al.*, 1972) and *Linaria* (CHARLTON, 1965); inflorescence sections of *Haworthia* (MAJUMDAR, 1970); cotyledon of *Biota* (KONAR and OBEROI, 1965) *Ilex* (HU and SUSSEX, 1971) and *Lactuca sativa* (DOERSCHUG and MILLER, 1967; RONCHI and GIOVANNA, 1970).

The physiological age of the explant is another factor which exercises an influence on organ formation. For instance, *Echeveria* leaf explants that were young initiated only roots, whereas older leaves regenerated shoot buds, and leaves of medium age produced both shoots and roots (RAJU and MANN, 1970). Rooting occurred only in young petioles of *Lunaria* (PIERIK, 1967, 1972).

Seasonal variations exercised a profound influence on regeneration. Explants of the December and April batch of *Solanum tuberosum* were highly tuberogenic while those obtained in February–March or May–November showed poor response (FELLENBERG, 1963).

Oxygen gradient in a tissue culture may play an effective role in the promotion of organogenesis as confirmed by KESSEL and CARR (1972) in carrot. With reduction in the available oxygen, shoot formation was favored while rooting required an increased oxygen gradient.

The quality and intensity of light often played a key role in organogenetic phenomenon (WEIS and JAFFE, 1969). The blue region of the spectrum promoted shoot formation and red light favored rooting (LETOUZL and BEAUCHESNE, 1969). Hence the nature of differentiation in a tissue can be controlled by exposure to light of different wave lengths. Light enhanced rooting in some isolated tissues and inhibited in others.

Embryos have been induced in *Nicotiana tabacum* var. Samsun callus cultures exposed to high intensity light 10000–15000 lux (HACCIUS and LAKSHMANAN, 1965, 1969) and adventitious buds in carrot callus among the numerous embryos arising from the periphery of small callus clumps or preformed proembryonal masses (HACCIUS, 1973).

Temperature, photoperiod, light intensity, pH, sugar concentration and even quality of agar are other factors that may have a determining role in organogenesis and embryogenesis but on which the data are meagre. Lilies and gladioli are temperature-sensitive and so are the tissue cultures derived from them. Low temperature and chilling of explants prior to culture favored their regenerative potential. There is also enough evidence to indicate that the effect of environmental factors such as light and temperature (PIERIK, 1967; GAUTHERET, 1971) are mediated by changes in the endogenous levels of various plant growth regulators, and perhaps synthesis of new ones such as the hypothetical rhizocaline and phyllocaline which act in consort with sugars in morphogenesis.

7. Somatic Embryogenesis

The fertilized egg cell (zygote) develops into an adult organism passing through different stages of embryogenesis. Though a somatic cell contains the same genetic material, it normally does not do so in isolation. Diffusion or physiological gradients of substances must occur in cell clusters if organ formation or embryogenesis is to be initiated as shown by ROSS and THORPE (1973). Somatic tissue of carrot has shown evidence of embryo initiation in single cells that are progenitors of bipolar embryos (REINERT, 1959; LINSER and NEUMANN, 1968; BACKS-HÜSEMANN and REINERT, 1970). In this instance the balance of hormone levels led to the differentiation of embryos. STEWARD *et al.*, (1964) and HALPERIN and WETHERELL (1964) observed that tissue derived from carrot embryos spontaneously differentiated into embryos. Plant extracts and other growth adjuvants were particularly active in causing cells to undergo an essentially embryoidal type of development. HOMÈS (1967) showed that embryogenesis can be induced on a purely synthetic medium containing mineral salts, aneurin, glucose and IAA (10^{-5}M) so that liquid endosperm of coconut was not a prerequisite for inducing embryony.

Embryo differentiation is considerably influenced by the physiological state of the calli themselves and the carry-over effects of auxins from inoculum to subculture. For instance, 2,4-D in the medium is least conducive for either organogenesis as in *Brassica oleracea* (LUŠTINEC and HORÁK, 1970) or embryogenesis as in carrot (REINERT and BACKS, 1968) but then it is possible that its effects are more pronounced when applied in sequence (REINERT and TAZAWA, 1969). HALPERIN (1970) has shown that embryogenesis could be induced only in those suspension cultures which are derived from explants grown on an auxin-containing medium. Nutrient media used for the initial proliferation of the tissue played a vital role in inducing embryogenesis. Cytokinins and gibberellins caused a partial or complete inhibition of potentially embryogenic cells.

In many instances, it was not necessary to dissociate plant tissue into single cells in order to achieve embryogeny contrary to the postulation by STEWARD.

The form in which nitrogen is supplied to the tissue system is another key factor in the determination of embryogeny. HALPERIN and WETHERELL (1965b) have observed that ammonium ions and casein hydrolysate at low levels are strongly stimulatory to embryogenesis in comparison to nitrate.

8. Suspension Culture: Growth in Liquid Media

Living cells from the mesophyll tissue of leaves could be isolated on a sufficiently large scale and suspended in liquid medium to permit their utilization in physiological and morphogenetic studies. Callus forms yet another source of free cells. When callus is grown in an agitated liquid medium, it forms a suspension composed of free cells and cellular aggregates ranging from a very few to several hundred cells, depending on the friability of the tissue.

Agitation can be achieved by using different culture vessels and different shaking conditions such as Auxophyton (the Steward apparatus), orbital shakers, spinning cultures and stirred cultures. During the period of culture, there is a peak in mitosis at about seven days after culture followed by a gradual fall in frequency after two weeks culminating in a complete cessation of mitosis in three weeks (TORREY *et al.*, 1962). Increase in cellular material reaches a saturation stage, beyond which there is no further increase. If such a saturated suspension is subcultured by diluting it back to the initial cellular contents, the same growth cycle is repeated. Movement of the liquid medium facilitates fragmentation of tissue leading to smaller units and helps in their gaseous exchange. Aggregates of cells divide more often as compared to free cells resulting in an increase in the size of the cell clusters in the earlier part of the growth cycle. At this stage the number of free cells remains constant. With ageing of culture, an increase in the frequency of free cells is observed resulting from the sloughing off of free cells from the cellular aggregates but not preceded by mitosis. There is no increase in wet weight or dry weight of the tissue in correlation with the peak in mitosis, but a marked increase in both was observed in the later period, during which the cells enlarged and differentiated. The free cells senesce and die if they do not divide further.

A suspension composed entirely of free cells like bacterial suspension, is desirable but hard to obtain. Tissues differ in the extent to which they are dispersed in a liquid medium. *Antirrhinum* suspension cultures contained aggregates up to 4 mm in diameter with very few free cells (MELCHERS and BERGMANN, 1958), while Paul's scarlet rose suspensions were made up of a high frequency of free cells (LIAU and BOLL, 1970). A free cell suspension devoid of cellular aggregates is possible only if there is a change in the chemical nature of the middle lamella of cell walls so that the daughter cells separate out before the onset of the next division. Such a mutant is still to be obtained. Several other factors can affect the degree of cell separation to a certain extent. REINERT and WHITE (1956) observed that withdrawl of folic acid and vitamin B_{12} from the cultures media of *Picea glauca* increased the friability of the tissue. Cell separation is promoted by high auxin concentration, an appropriate balance between auxin and yeast extract, between auxin and coconut milk or between auxin and kinetin. In *Sycamore* suspension cultures lowering of the coconut milk concentration and increasing the 2,4-D concentration gave a more pipetteable cell suspension (LAMPORT, 1964) whereas in *Atropa* a decrease in kinetin concentration from 0.5 mg/l to 0.1 mg/l gave a better yield in cell separation (DAVEY *et al.*, 1971). Frequent subculturing at the exponential phase will lead to an increase in the number of cell aggregates rather than the number of free cells. Mineral nutrients in the medium like ratio of K/Mg and levels of Ca may change the friability of the callus (YOSHIDA and WATANABE, 1971). Incorporation of cell wall degrading enzymes such as macerozyme (0.05%) and cellulase (0.1%) enhances cell separation without affecting the viability of the cells.

Great variation is observed in size and shape of the cells present in a suspension. The cells may be spherical (12–40 μ in diameter), slipper-shaped (30–60 × 10–20 μ) and gourd-shaped (95 × 40 μ) as in *Phaseolus* or may frequently become multinucleate and giant-sized (300 μ diam. and 2.5 mm long) as in tobacco. Chromosome changes may occur in cells leading to euploid or aneuploid numbers.

9. Growth of Isolated Single Cells

Three methods have been employed for the study of growth of single cells in culture. In the first, the nurse-raft technique, isolated cells are placed on a filter paper over a callus tissue which acts as a nurse (MUIR *et al.*, 1954, 1958). The nurse tissue supplies all the metabolites necessary for inducing and maintaining cell division of the isolated cell. The method of microculture involves culture of cells in a microchamber as described by JONES *et al.* (1960). Though each cell is capable of synthesizing all the metabolites it requires, they are lost to the medium by diffusion as the cells are usually "leaky" but serve to "condition" the medium, thereby stimulating the cell to divide.

In the plating technique, filtered suspension containig a known number of cells is mixed with a thin film of agar (BERGMANN, 1960) in a Petri plate. Even distribution of the cell suspension as a thin layer over agar yields good results. Petri plates are sealed by parafilm to prevent drying, before being kept for incubation. At appropriate plating density, concentrations of metabolites external to the cell reach an optimum which limits the net efflux, and the cell is capable of maintaining its intracellular concentration. At this stage, the cell undergoes division and forms colonies.

In a plated suspension, the division first starts in small cellular aggregates and then in individual cells. Usually the highest plating efficiency is obtained from exponentially growing cultures which contain more cellular aggregates than single cells. Plating efficiency does not reach the theoretical 100%. The density of plated cells is one of the key factors determinative in cell division.

The least minimal density so far used is $1.0–1.25 \times 10^3$ cells/ml. Clones arising from single cells may be of mixed origin, both from the aggregates as well as single cells. Use of cell wall-degrading enzymes in suspension and subsequent plating will lead to clones mostly of single cell origin. Recent experiments show that condition of the plated atmosphere is important in determining plating efficiency. In a specially designed suspension culture vessel, a low density suspension containing 600 cells/ml can be made to grow into an actively growing suspension by exposing it to an atmosphere from a profusely dividing suspension. The CO_2 present in such an atmoshpere induced growth in low density cell suspension. Hence it is possible to trigger cell division in such suspensions by enhancing the CO_2 present in its immediate environment. On the other hand, an increase in the "energy state" at the critical region of dissolved O_2 could stimulate activity of new or existing enzymes towards embryo-like developments as in carrot tissue (KESSELL and CARR, 1972).

A proper adjustment of pH and raising the level of iron may improve plating efficiency. In *Sycamore* callus (STUART and STREET, 1971) pH adjustment of the medium to 6.4 reduced the minimum effective level of cell density from $9–15 \times 10^3$ cells/ml to 2×10^3 cells/ml.

Application of low concentration of ethylene (2.5 mg/l) showed a promotive effect on growth. An appropriate balance of auxin to cytokinin is another factor controlling cell division. The requirements of the plated suspension will be different from that of the routinely subcultured callus. For *Sycamore* cells, a medium

containing GA_3, cytokinin and amino acids is essential, though none of them are required for callus growth.

The developing clones may differ in their appearance, texture, friability, pigmentation, growth potential, metabolic requirements, resistance to chemicals and antibiotics, and also in their totipotency. They may show differences in ploidy. Clones of tobacco differing in their auxin and cytokinin requirements have also been isolated (FOX, 1963). Suspension cultures more resistant to acriflavine than the parental strain have been established in carrot (BLAKELY and STEWARD, 1964). DAVEY *et al.* (1971) obtained clones of *Atropa belladonna* differing in growth rate, nutrient requirement and chloroplast structure. Variations can be induced at the cellular level by treatment with chemical mutagens or radiations.

Resistant clones can be selected by plating in specific media containing the chemical. CARLSON (1973) selected cells resistant to methionine sulfoximine in an attempt to regenerate plants resistant to the pathogen *Pseudomonas tabaci*. Streptomycin-resistant mutants (MALIGA *et al.*, 1973) and BUdR-resistant cell lines of tobacco have thus been obtained. If it is some nutritional requirement, the knowledge that nutritionally deficient strains do not divide in a minimal medium can be utilized to advantage. Wild-type cells divide in a minimal medium and they can be made to incorporate BUdR—a base analog of thymidine in their DNA. On exposure of such a population containing wild-type and nutritionally deficient mutant cells to visible light, there is a selective destruction of wild cells, since the DNA-containing BUdR is sensitized. The mutant clones can then be analyzed for their nutritional requirement. CARLSON (1970) by the same procedure, isolated six auxotrophic mutants of haploid cells of tobacco which showed independent requirements for hypoxanthin, biotin, p-aminobenzoic acid, arginine, lysine and proline.

10. Embryogenesis in Free Cell Suspensions

Free cells can undergo cell division to form a callus mass, which under controlled conditions of growth is capable of initiating organ primordia de novo. Another type of organization most frequently observed in suspension cultures, is the development of embryos capable of forming complete plantlets passing through stages similar to those observed in normal embryogeny. The decisive nature of an embryo is its bipolarity with a shoot and a root pole at opposite ends. In callus cultures where embryos are formed, diffusion gradients in the tissue may determine polarity. In free-floating cells the stimulus which brings about polarity is difficult to explain. Single cells in isolation, however, do not directly transform themselves into embryos and become exhausted in the process. On the other hand, it is evident that potentially embryoidal cells are differentiated from amongst cellular aggregates (arising from divisions of isolated cells) as revealed by their differential staining properties, conspicuous size and the presence of a large number of nucleoli per nucleus (KONAR *et al.*, 1972b). These developmental activations involve synthesis of ribosomal RNA and protein (SUSSEX, 1972). The embryoidal cells in a clone are subject to gradients of growth substances in their

further totipotent expression. Isolated carrot cells have shown capacity to produce embryos (BACKS-HÜSEMANN and REINERT, 1970). By direct microscopic observation of the carrot cell undergoing embryogenesis, REINERT *et al.* (1971) reached the conclusion that a multicellular aggregate preceded embryo formation. But the pattern of early segmentations in such embryologically prone cells up to the globular embryo stage differed, at least in carrot, from the typical embryogenesis of an egg cell, although the resemblance was closer towards later stages of ontogeny (MCWILLIAM *et al.*, 1974).

The nutrient factors which initiate an essentially embryonal type of development have been widely studied. Transfer of tissue from an auxin-containing to an auxin-free medium, and addition of nitrogenous compounds such as amino acids, trigged embryogenesis (REINERT, 1959). Cell suspensions of *Atropa belladonna* developed embryos and plantlets on transfer to a medium containing tropic acid (THOMAS and STREET, 1972) in place of auxins. STEWARD (1963) presumed that conditions simulatory to the embryo sac are necessary for a cell to pass through embryogenesis. Liquid endosperm (coconut milk) may bring about conditions favorable for embryo formation but it has now been proved that embryogenesis can occur in a purely synthetic medium.

Physical or physiological isolation of a cell from the correlative influences of the adjacent tissue is also not a necessary prerequisite, as postulated by STEWARD, for recapitulating embryogenesis in vitro. The occurrence of protoplasmic strands connecting the potentially embryonic cells with the surrounding tissue disproved this concept (THOMAS *et al.*, 1972). BUTENKO *et al.* (1967) noted that mineral salts should be present at a high concentration for embryo initiation. Addition of nitrogen, either inorganic (KNO_3, NH_4NO_3) or organic (amino acids and amides) is conducive for the initiation of embryogenesis (TAZAWA and REINERT, 1969) but their concentrations vary. Glutamine at 5 mM added to White's basal medium which contained 3.2 mM inorganic nitrogen proved effective for embryogenesis, while higher concentrations of KNO_3 (40 mM) were necessary to achieve the same results. However, embryos were never formed with nitrate as a sole source of nitrogen at any concentration within the physiological range but was necessary for the development of advanced stages. The conversion of a somatic cell to an embryo may not be the outcome of an increased availability of nitrogen, but could as well be caused by an increased ratio of nitrogen to auxin (REINERT, 1973). The nitrogenous compounds are utilized for protein synthesis but how exactly they effect a change in gene expression deflecting a somatic cell from its normal course to the pathway of an embryo, is not known.

11. Morphogenetic Potential in Relation to Subculture

Many cultured tissues exhibit a high potential for organogenesis or embryogenesis when first initiated, but gradually a decline sets in as subculture proceeds with eventual loss of morphogenetic response. Several workers have observed this phenomenon in callus that had passed through several passages (TORREY, 1967;

MURASHIGE and NAKANO, 1967) and in free cell suspensions (SYŌNO, 1965; REINERT and BACKS, 1968; REINERT *et al.*, 1971; THOMAS and STREET, 1970, 1972; SMITH and STREET, 1974; see also Chap. III.1 of this Vol.).

The loss of morphogenetic potential in a tissue may be due either to a genetic or a physiological change induced by prolonged cultural conditions. In a genetic change, the chances of restoration are very low, while this can be achieved by the addition or substitution of phytohormones and nutrient constituents if the alteration is physiological.

The genetic effects in a cultured tissue are reflected in changes of chromosome number leading to euploidy or aneuploidy, or chromosome aberrations and mutations. Cultured tissue may show a chromosome number different from the parental strain (ZOSIMOVICH *et al.*, 1972; WRIGHT and NORTHCOTE, 1973; RAVKIN and POPOV, 1973). A correlation between changed ploidy and loss of regenerative potential has been noted in several tissues (TORREY, 1967; MURASHIGE and NAKANO, 1967; see also Chap. III.1 of this Vol.). According to SMITH and STREET (1974), subculture leads to cells of impaired or nil totipotency due to some changes in nuclear cytology, and such cells are at a selective disadvantage as compared to the normal cells. Eventually at later stages of culture, they are entirely replaced by nontotipotent cells. An alteration in karyotype need not always result in morphogenetic incapability as for instance, regeneration of extreme aneuploid plants from 20-year-old tobacco tumor tissues has been observed (SACRISTÁN and MELCHERS, 1969). According to SYŌNO and FURUYA (1972) plantlets regenerated from old subcultures were weak and abnormal and failed to develop into mature plants. NORIKO (1972) noted that organogenesis could not be induced in the early stages of tobacco callus culture, while in the later part of the same subculture (stationary phase and later), the potential for organogenesis could be enhanced. In such cultures during the initial stages, the cells showed enlarged nuclei and were often multinucleate, while during the stationary phase, the size and shape of the nuclei became more or less uniform. Such cells were capable of embryogenesis.

According to an alternative hypothesis, subculture often leads to a loss of many endogenous factors present at the critical stages of growth. Such factors (morphogens) occurring in the primary callus cultures may not be synthesized at all or synthesized only in insufficient quantity at later stages. Hence if these are added to subcultured tissue, then restoration of morphogenetic potential should be possible. WOCHOK and WETHERELL (1972) reported that addition of kinetin could restore decline in regenerative response in long-term carrot cultures, while in primary stages no promotive effect of the chemical was observed. The gradual loss of requirement for exogenous auxins (habituation) has been reported frequently. Many of these altered strains produced auxins and cytokinins endogenously. Based on their studies of restoration of embryogenesis in long-term cultures of carrot by a change in the composition of the culture medium, REINERT *et al.* (1971) agreed with the physiological hypothesis.

It is probable that both the processes are involved in the decline and loss of morphogenetic response during prolonged culture. Alterations may also be brought about in nuclear cytology leading to nondiploid cells in a callus. Some of these cells may be eliminated, others may become stable components of a mixed

population, while still others may have a selective advantage over normal cells. If nutrient factors supplied to a mixed population containing totipotent and nontotipotent cells are able to effect the growth of totipotent cells selectively, then restoration can be said to have been achieved. On the other hand, if such impaired cells predominate and are selected as subculture proceeds, the result will be a population composed entirely of such altered cells, rendering them incapable of morphogenesis even by a change of the nutrient and other conditions. Such aspects controlling organogenesis and embryogenesis have been discussed at length by REINERT (1973) and SMITH and STREET (1974).

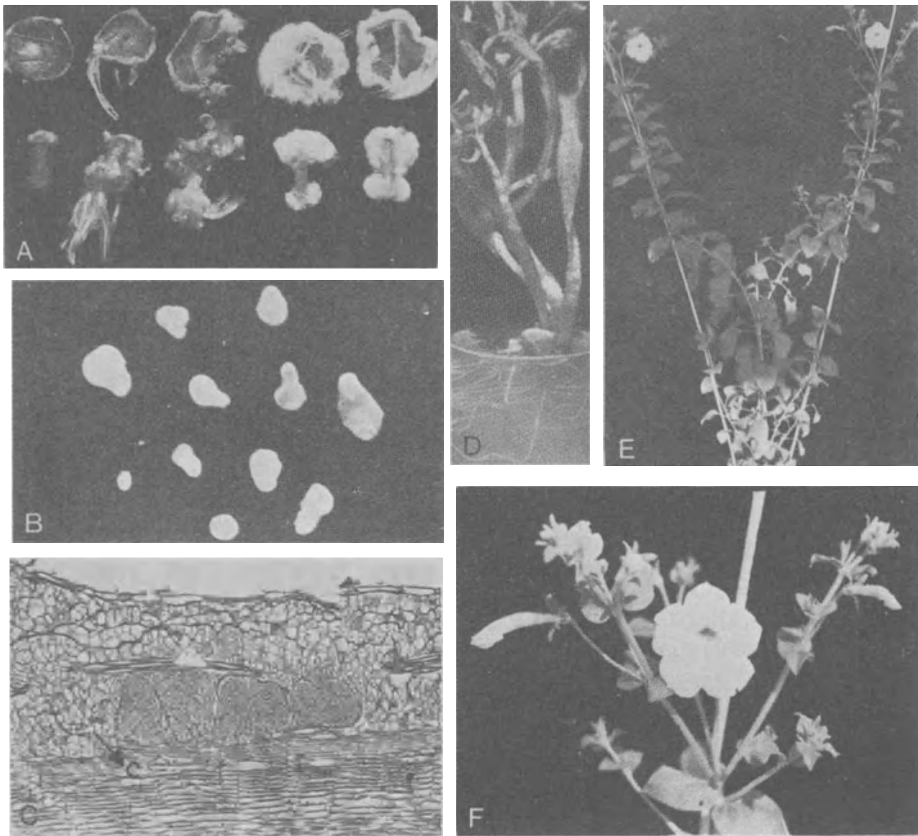

Fig. 1A–F. Induction of shoot buds/embryos in *Petunia* tissue cultures. (A) Morphogenetic responses of stem and leaf explants of *Petunia inflata* to various growth substances. *Left to right:* Control, root formation with IAA (1 mg/l); callus and bud development with BA (200 μg/l); abundant root formation with NAA (1 mg/l); callus and root development with NAA (1 mg/l) + BA (200 μg/l). × 2. (B) Somatic embryo isolated from 4-week-old callus grown on MS + 2,4-D (1 mg/l) medium. × 5. (C) Section showing initiation of embryonal groups from cambial *C* region. × 40. (D) Young plantlet from somatic embryo. × 1.5. (E, F) Plantlet bearing normal flowers, (A, B) after RAO *et al.*, 1973a; (C) after HANDRO *et al.*, 1973b, (D–F) after RAO, unpublished

12. Asexual Propagation in Herbaceous Angiosperms

Many herbaceous annuals and perennials have responded favorably to the aseptic manipulation of cells and tissues derived from them in efforts aimed at regenerating complete plantlets through organogenesis or embryogenesis for clonal multiplication. Micropropagation of plants represents an alternative method to sexual reproduction whereby whole plants are obtained not from seeds but from cells of somatic or gametic origin (Fig. 1). Breeding for a "plant ideotype" is a postulation in agriculture and cell totipotency has been exploited to advantage in attempts at neoformation of plants on a mass scale. That rapid progress has been made in this direction during the past few years, is reflected in the immense success achieved with many of the ornamentals and economically important plant species. Development of plantlets through shoot buds/embryos has been realized in the following plant species:

Table 2a. Herbaceous dicotyledonous angiosperms: ornamentals

Plant	Nature of regeneration	Reference
Anagallis arvensis L.	Adventitious buds and embryos	BAJAJ and MÄDER (1974)
Antirrhinum majus L.	Embryos	POIRIER-HAMON *et al.* (1974)
Arabidopsis thaliana (L.) Heynh.	Embryos and plantlets	CORCOS (1973)
Asclepias curassavica L.	Embryos	PRABHUDESAI and NARAYANASWAMY (1973)
Begonia rex Putz.	Shoot buds and roots	CHLYAH (1972)
Begonia × *Cheimantha*	Plantlets	FONNESBECH (1974)
Codiaeum variegatum Blume	Shoot buds	CHIKKANNAIAH and GAYATRI (1974)
Consolida orientalis (Gay) Schrod.	Shoot buds	NATARAJA (1971)
Chrysanthemum 8 sp.	Shoot buds and plantlets	WATANABE *et al.* (1972)
C. cinerariaefolium Vis.	Adventitious buds and plantlets	ROEST and BOKELMANN (1973)
C. morifolium Ramat	Adventitious buds and plantlets	EARLE and LANGHANS (1974a)
Crepis capillaris (L.) Wallr.	Shoot buds and plantlets	JAYAKAR (1970); YAZAWA (1972)
Convolvulus arvensis L.	Shoot buds and plantlets	BONNETT and TORREY (1965); HILL (1967a)
Cyclamen persicum Mill.	Shoot buds and roots	STICHEL (1959); LOEWENBERG (1969)
Dianthus sp.	Shoot buds and plantlets	PETRŮ and HANDA (1974); HACKETT and ANDERSON (1967)
Eschscholtzia californica Champ.	Embryos	KAVATHEKAR and GANAPATHY (1973)
Euphorbia pulcherrima Wild.	Shoot buds	NATARAJA *et al.* (1973); DE LANGHE *et al.*(1974); NATARAJA (1975)
Gerbera jamesonii Bolus	Shoot buds and plantlets	PIERIK *et al.* (1973); MURASHIGE *et al.* (1974)

Table 2a. (continued)

Plant	Nature of regeneration	Reference
Helianthus annuus L.	Shoot buds	SADHU (1974)
Kalanchoe pinnata Pers.	Shoot buds, embryos and plantlets	WADHI and MOHAN RAM (1965)
Lunaria annua L.	Shoot buds	PIERIK (1967)
Montbretia crocosmaeflora Lemoine	Shoot buds and plantlets	MATSUZAWA and SATO (1972)
Mesembryanthemum floribundum Haw.	Shoot buds and embryos	MEHRA and MEHRA (1972)
Macleaya cordata R. Br.	Embryos	KOHLENBACH (1965)
Mazus pumilus Loor.	Shoot buds	RASTE (1971)
Nigella damascena L.	Embryos and shoot buds	RAMAN and GREYSON (1974); BANERJEE and GUPTA (1975)
Passiflora coerulia L.	Shoot buds, roots and embryos	NAKAYAMA (1966)
Pelargonium hortorum Bailey	Shoot buds and plantlets	CHEN and GALSTON (1967); PILLAI and HILDEBRANDT (1969)
Petunia hybrida cv. Cascade and cv. Rose du Ciel	Shoot buds, embryos and plantlets	RAO *et al.* (1973a, b)
Petunia inflata R. Fries	Shoot buds, embryos and plantlets	HANDRO *et al.* (1973a, b)
Petunia axillaris (Lam.) BSP	Shoot buds and plantlets	SWAMY and CHACKO (1973)
Phlox drummondii Hook.	Shoot buds	KONAR and KONAR (1966)
Ranunculus sceleratus L.	Embryos and plantlets	KONAR and NATARAJA (1970); NATARAJA and KONAR (1970)
Rosa sp.	Shoot buds	HILL (1967b) JACOBS *et al.* (1968)
Salpiglossis sinuata L.	Shoot buds and plantlets	HUGHES *et al.*, (1974)
Saintpaulia ionantha Wandt.	Shoot buds and plantlets	KUKULCZANKA and SUSZINSKA (1972)
Sinapis alba L.	Shoot buds and embryos	BAJAJ and BOPP (1972)
Scopolia parviflora Nakai	Shoot buds	TABATA *et al.* (1972)
Taraxacum officinale Wigg.	Shoot buds	BOWES (1970)
Torenia fournieri Lind	Shoot buds and plantlets	BAJAJ (1972)
Trifolium repens L.	Plantlets	PELLETIER and PELLETIER (1971)
Trigonella foenumgraecum L.	Shoot buds	SUBRAMANIAM *et al.* (1968)
Vigna unguiculata (L.) Walp.	Shoot buds	SUBRAMANIAM *et al.* (1968)

13. Regeneration in Cereal Tissue Cultures

Very recently, various millets and cereals have been brought into culture as callus and complete plantlets redifferentiated from their respective calli, following sequential application of plant hormones i.e., transfer from a high auxin-containing medium to an auxin-omitted medium. Regenerated plantlets of *Panicum*, *Paspalum*, *Pennisetum* and *Eleusine* (RANGAN, 1973, 1974) could be transferred to soil and grown to maturity yielding grains (Fig. 2).

Table 2b. Plants of economic value

Plant	Nature of regeneration	Reference
Ammi majus L.	Embryos	SEHGAL (1972)
Anethum graveolens L.	Embryos and plantlets	JOHRI and SEHGAL (1965); SEHGAL (1968); RATNAMBA and CHOPRA (1974)
Apium graveolens L.	Embryos	REINERT *et al.* (1966)
Asparagus officinalis L.	Embryos, shoot buds and plantlets	WILMAR and HELLENDOORN (1968); TAKATORI *et al.* (1968); MALNASSAY and ELLISON (1970); YAKUWA *et al.* (1971); STEWARD and MAPES (1971b); HARADA (1973); JULLIEN (1974); YANG and CLOSE (1974); BUI-DANG-HA *et al.* (1975);
Atropa belladonna L.	Embryos, shoot buds and plantlets	THOMAS and STREET (1970)
Beta vulgaris L.	Shoot buds and plantlets	ATANASOV and KIKINDONOV (1972); BUTENKO and ATANASOV (1971) BUTENKO *et al.* (1972)
Brassica oleracea L. var. *Botrytis*	Shoot buds	LUSTINEC and HORÁK (1970); BARONCELLI *et al.* (1973)
B. oleracea L. var. *gemmifera* D.C.	Shoot buds and plantlets	CLARE and COLLIN (1974)
B. oleracea L. var. *capitata*	Shoot buds and plantlets	BAJAJ and NIETSCH (1975); HORÁK *et al.* (1975)
B. napus var. Zephyr	Shoot buds and plantlets	KARTHA *et al.* (1974)
Carum carvi L.	Embryos	AMMIRATO (1974)
Conium maculatum L.	Embryos with cotyledons, hypocotyl and pseudo roots	NÉTIEN and RAYNAUD (1972)
Coptis japonica Makino	Plantlets	SYŌNO and FURUYA (1972b)
Coriandrum sativum L.	Embryos	STEWARD *et al.* (1970)
Cichorium endivia L.	Shoot buds, embryos and plantlets	VASIL and HILDEBRANDT (1964, 1966a)
C. intybus L.	Shoot buds and plantlets	BOUNIOLS and MARGARA (1968); BOURIQUET (1972)
Cucurbita pepo DC	Embryos, shoot buds and plantlets	SCHROEDER (1968); JELASKA (1974)
Datura innoxia L.	Shoot buds	ENGVILD (1973)
Daucus carota L.	Embryos and plantlets	REINERT (1959); KATO and TAKEUCHI (1963); STEWARD *et al.* (1964); HALPERIN and WETHERELL (1965a); HOMÈS and GUILLAUME (1967)
Dioscorea sansibarensis Pax.	Limited shoot buds	RAO (1969)
Fagopyrum esculentum Moen.	Shoot buds and plantlets	YAMANE (1973)
Foeniculum vulgare Goert.	Embryos	MAHESHWARI and GUPTA (1965)
Glycine max. (L.) Merrill.	Shoot buds	KIMBALL and BINGHAM (1973)

Table 2b. (continued)

Plant	Nature of regeneration	Reference
Ipomoea batatas (L.) Lam. and Poiret	Shoot buds (rooting followed by shoots)	NAKAJIMA and KAWAKAMI (1969); GUNCKEL *et al.* (1972); YAMAGUCHI and NAKAJIMA (1972)
Lactuca sativa L.	Shoot buds	DOERSCHUG and MILLER (1967)
Lycopersicon esculentum Mill.	Shoot buds and plantlets	PADMANABHAN *et al.* (1974)
Lotus corniculatus L.	Embryos	NIIZEKI and GRANT (1971)
L. caucasicus Kupr.	Embryos	NIIZEKI and GRANT (1971)
Medicago sativa L.	Shoot buds and plantlets	SAUNDERS and BINGHAM (1972)
Nicotiana sp.	Shoot buds and plantlets	vide MURASHIGE (1974)
Petroselinum hortense Hoffm.	Embryos and plantlets	VASIL and HILDEBRANDT (1966b)
Pergularia minor Andr.	Shoot buds, embryos and plantlets	PRABHUDESAI and NARAYANASWAMY (1974)
Pisum sativum L. cv. Century	Shoot buds and plantlets	GAMBORG *et al.* (1974)
Pogostemon cablin Benth.	Shoot buds and plantlets	HART *et al.* (1970)
Rauvolfia serpentina Benth.	Embryos and plantlets	MITRA and CHATURVEDI (1970)
Sium sauve Walt.	Embryos	STEWARD *et al.* (1970)
Solanum melongena L.	Embryos and plantlets	YAMADA *et al.* (1967)
S. tuberosum L.	Shoot buds and plantlets	OKAZAWA *et al.* (1967) BAJAJ and DIONNE (1967); SKIRUIN *et al.* (1975)
S. xanthocarpum L.	Shoot buds	RAO and NARAYANASWAMY (1968)
Tylophora indica Merr.	Embryos and shoot buds	RAO *et al.* (1970); RAO and NARAYANASWAMY (1972)

Table 2c. Bulb plants

Plant	Nature of regeneration	Reference
Allium cepa L. var. proliferum	Shoot buds and plantlets	FRIDBORG (1971)
Allium sativum L.	Shoot buds and plantlets	HAVRÁNEK and NOVÁK (1973)
Aloe pretoriensis Pole Evans	Shoot buds and plantlets	GROENWALD *et al.* (1975)
Anthurium andraeanum Lind.	Shoot buds and plantlets	PIERIK *et al.* (1974)
Cymbidium sp.	Protocorms	FONNESBECH (1972)
Epidendrum sp.	Plantlets	CHURCHILL *et al.* (1973)
Freesia sp.	Shoot buds and plantlets	BAJAJ and PIERIK (1974)
Gladiolus hortulans L.	Shoot buds and plantlets	ZIV *et al.* (1970); SIMONSEN and HILDEBRANDT (1971)
Haworthia variegata Haw.	Shoot buds and plantlets	MAJUMDAR (1970); KAUL and SABHARWAL (1972)
Hippeastrum hybridum Hort.	Buds and roots	MII *et al.* (1974)
Hyacinthus orientalis L.	Bulblets	PIERIK and WOETS (1971); TAMURA and KABE (1971); SANIEWSKI *et al.* (1974)
Laeliocattleya sp.	Shoot buds	CHURCHILL *et al.* (1973)
Lilium longiflorum Thunb.	Shoot buds and plantlets	SHERIDAN (1968)
L. speciosum Thunb.	Shoot buds and bulblets	ROBB (1957)
L. regale L	Shoot buds and plantlets	MONTEZUMA-DE-CARVALHO *et al.* (1974)

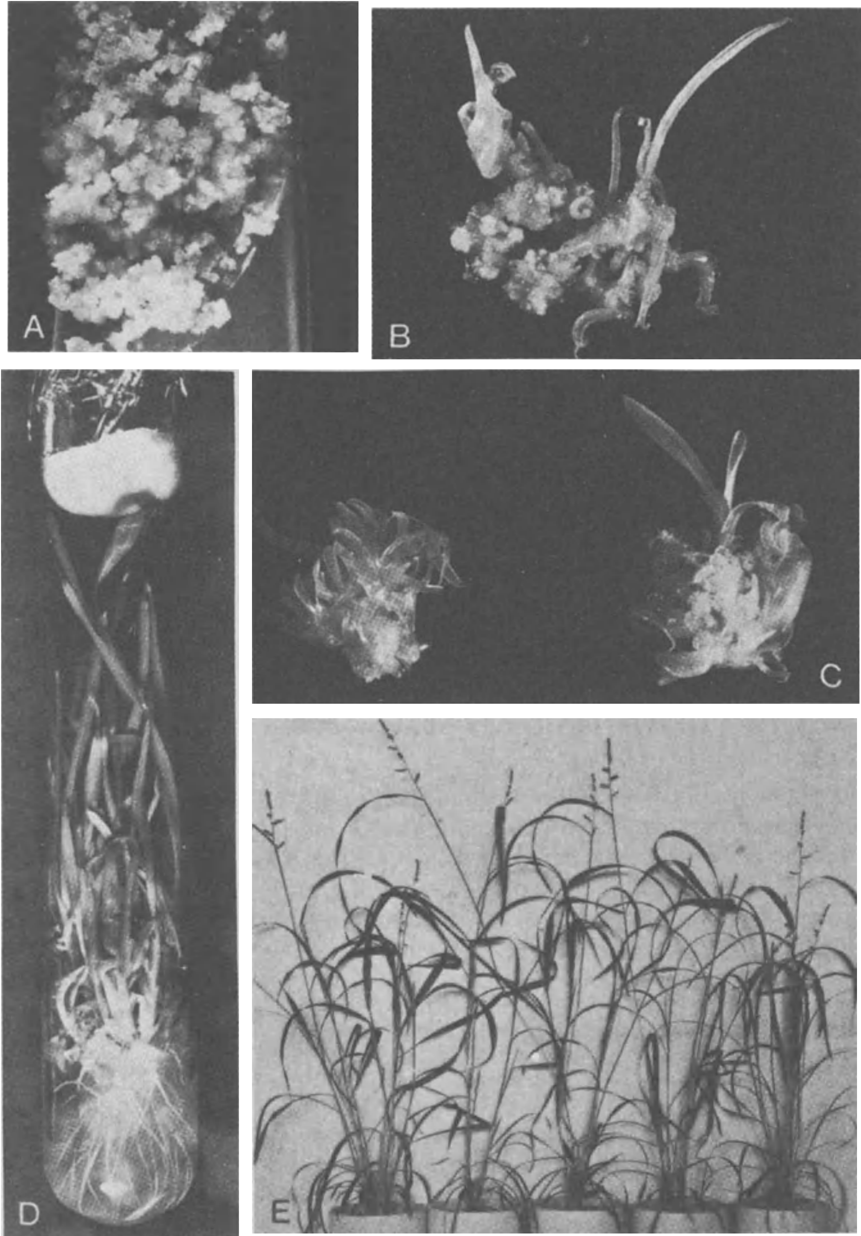

Fig. 2A–E. Regeneration of plants in millet tissue cultures. (A) *Paspalum scrobiculatum*: 12-week-old callus culture (on MS + 15% CM + 10 mg/l 2,4-D). × 1.2. (B) Shoot bud induction in *Paspalum serobiculatum* callus culture (on the same medium but with 0.2 mg/l of NAA). × 1.5. (C, D) Regeneration in callus cultures of *Eleusine coracana* (on medium similar to *P. scrobiculatum*). (C) × 1.5; (D) × 0.8. (E) Tissue culture-induced plants of *Panicum miliaceum* reared to flowering stage. × 0.2. (A–D) after RANGAN, unpublished; (E) after RANGAN, 1974

Tissue cultures of maize, wheat and sorghum established from seedling segments have been grown for nearly 10 years on nutrient media (MASCARENHAS *et al.*, 1975a, b). Cultures of these cereals grew as a mixture of callus and roots without loss of vigor or rooting potential. According to these authors, the reversal of callus to roots, and of roots to callus could be repeated several times by transfer to agar or agitated liquid medium. Abscisic acid showed a striking effect in suppressing growth of roots, but was inconsequential for callus growth.

Plantlets could be regenerated from explanted tissues containing the growing tip in the case of maize (var. Golden Sunshine) and *Sorghum vulgare*, where a medium supplemented with edamin, inositol and NAA was highly favorable, but regenerants of sorghum did not survive for more than 30 days. Mesocotyl region of the seedling, mediated through callus formation, was particularly regenerative in the case of wheat and rice (HENDRE *et al.*, 1974). Organogenesis from callus cultures of oats (CARTER *et al.*, 1967) and barley (CHENG and SMITT, 1975) has been realized.

Commercial possibilities of sugarcane plants obtained in substantial numbers through reconstitution of plantlets from callus (HEINZ and MEE, 1969) and from cell cultures of 'mosaic' plants are being explored (BARBA and NICKELL, 1969; NICKELL, 1973). For details on tissue culture of cereals, see Chapters I.8. and II.3,4. of this volume.

14. Organogenesis in vitro as Applicable to Forest Tree Propagation

In recent years tissue culture techniques have been increasingly applied to tree-breeding programs with the ultimate idea of rearing plantlets by the hundreds, capable of being transplanted to soil. Induction of organogenesis/embryos has been attempted in somatic callus cultures of quite a large number of deciduous trees such as birches, beeches, aspens, poplars and elms, conifers and woody dicot angiosperms (vide WINTON, 1972a, b), but few woody tissues have shown large-

Table 3. Tree species of economic importance capable of organogenesis/embryogenesis in vitro

Plant	Nature of regeneration	Reference
1. Dicotyledons		
Azadirachta indica A. Juss.	Shoot buds	RANGASWAMY and PROMILA (1972)
Carica papaya L.	Embryos and plantlets	DE BRUIJNE *et al.* (1974)
Citrus sp.	Embryos and plantlets	RANGASWAMY (1961); MURASHIGE (1974)
Coffea arabica L.	Embryos and plantlets	SHARP *et al.* (1973)
C. canephora Pierre ex Roehner	Embryos	STARITZKY (1970a)
Coffea liberica Hiern	Embryos	STARITZKY (1970a)
Eucalyptus citriodora Hook.	Shoot buds and plantlets	ANEJA and ATAL (1969)
E. viminalis L. *E. obliqua* l'Herit.	Shoot buds and plantlets	BLAKE (1972)
Eremocitrus glauca Swing	Embryos	MURASHIGE (1974)

Table 3. (continued)

Plant	Nature of regeneration	Reference
Fortunella crassifolia Swing.	Embryos	MURASHIGE (1974)
Hevea brasiliensis Mull.-Arg.	Embryos and shoot buds	PARANJOTHY (1974); WILSON (1974)
Ilex aquifolium L.	Embryos on cotyledons	HU and SUSSEX (1971)
Microcitrus australasica F. Muel.	Embryos	ESAN (1973)
Microcitrus warburgiana F. M. Bail	Embryos	ESAN (1973)
Poncirus trifoliata L.	Embryos	SINGH (1963)
Panax ginseng C. A. Mey.	Embryos	BUTENKO *et al.* (1968)
Prunus amygdalis L.	Shoot buds and plantlets	MEHRA and MEHRA (1974)
Populus × *Canadensis*	Plantlets	BERBEE *et al.* (1972)
Populus tremuloides Minchx.	Plantlets	WINTON (1968, 1970)
P. tremula L.	Plantlets	WINTON (1971)
P. nigra L. 'Italica'	Single leaves, buds or elongating shoots	GHUGALE *et al.* (1971); VENVERLOO (1973)
P. trichocarpa L.	Plantlets	BAWA and STETTLER (1972)
2. Monocotyledons		
Elaeis guineensis Jacq. var. dura Bece	Embryos and plantlets	RABÉCHAULT *et al.* (1972); JONES (1974)
3. Conifers		
Biota orientalis Endl.	Embryos	KONAR and OBEROI (1965)
Cryptomeria japonica D. Don	Shoot buds and roots	ISIKAWA (1974)
Picea abies L.	Leafy shoots	HUHTINEN quoted by WINTON (1974)
P. glauca (Moench) Voss.	Embryos	DURZAN *et al.* (1973)
Pinus banksiana Lamb.	Embryos	DURZAN and STEWARD (1970)
P. cembra L. *P. lambertiana* Dougl.	Shoots and roots	BERLYN quoted by WINTON (1974)
P. strobus L.	Embryos	ISIKAWA *et al.* (1973); DURZAN *et al.* (1973)
P. taeda L.	Embryos	SOMMER and BROWN (1974)
P. palustris Mill.	Embryos and plantlets	SOMMER *et al.* (1975)
P. elliotti (Slash pine)	Embryos and plantlets	SOMMER and BROWN (1974)
P. echinata Mill.	Embryos	vide WINTON (1974)
P. ponderosa Dougl.	Shoots and roots (nonfunctional)	vide WINTON (1974)
P. contorta (Shore pine)	Embryos	vide WINTON (1974)
Pseudotsuga menziesii (Douglas-fir).	Rooted and non-rooted embryos	vide WINTON (1974)

scale propensity for regeneration of either shoot buds or embryos apart from rooting (Table 3).

Shoot buds arising from subcultured stem callus of *Broussoneta kazinioki* (paper mulberry) could be made to root (OKA and OHYAMA, 1972) but their growth on soil remains to be ascertained as also the regenerative potential in species of *Acer*, *Avocado*, *Olea*, *Ulmus* and others. The only report of successful regeneration of complete plantlets through embryos in a monocot tree tissue culture is that of the oil palm *(Elaeis quineensis)* by RABÉCHAULT *et al.* (1972).

Tissue cultures derived from the isolated nucellus of ovules of *Citrus* and allied genera (RANGASWAMY, 1961; MURASHIGE, 1974) formed yet another source of experimental material for obtaining regeneration. Even the ovary wall tissue of unpollinated ovaries of *Citrus sinensis* and *C. aurantifolia* (MITRA and CHATURVEDI, 1972) and of stem callus of *C. grandis* (CHATURVEDI *et al.*, 1974) could differentiate embryos and plantlets.

The potential of plant endosperm for organogenesis in culture has been investigated by JOHRI and his associates (see Chap. III.4. of this Vol.) with partial success. Corn (STRAUS, 1960) barley, wheat (SEHGAL, 1969, 1974) and rye grass (NORSTOG, 1956) endosperm could grow in culture as callus, but lacked the potential for organogenesis.

15. Morphogenetic Potential of Higher Plants in vitro: Problems and Prospects

That a large number of plant species have been successfully brought into culture during the past decade indicated that establishment of a callus culture presents no problem. Efforts at regenerating whole plants in cultured tissue through the differentiation of shoot buds or embryos (see BAJAJ and BOPP, 1971) especially among the ornamentals such as *Petunia* (Fig. 1A–F), have also proved rewarding. The degree of regeneration varied considerably from species to species. Among those that have been highly regenerative are members of the family Solanaceae, Umbelliferae, Cruciferae, Compositae and a few of the Leguminoseae, comprising mostly herbaceous forms. Many are predisposed to form embryos or shoot buds while in those that do not, the causative factors are obscure. But application of tissue culture techniques as a practical means of plant propagation to agricultural crops has not yet been realized to the fullest extent.

Various millets and cereals among the crop plants, with the possible exception of oats, wheat and barley which appear to be low in morphogenetic potential, have shown surprising ability for large-scale regeneration through shoot bud formation capable of being transplanted to soil (see Chaps. I.8. and II.3. of this Vol.). Plants have also been successfully regenerated from cell cultures of sugarcanes that are chromosomal mosaics, and could as well be applied to crop plants such as rice and other cereals as a means of recovery of variant types through selection at the cellular level. Large-scale cultivation of tissue culture-induced sugarcane varieties, is being explored (NICKELL and HEINZ, 1973; Chap. I.1. of this Vol.) on the basis of yield and disease resistance. Such approaches promise exciting possibilities in asexual crop improvement.

It has been observed, however, that the frequency of plant development through callus cultures is relatively low in many of the economically important crop species and appallingly poor in most of the woody forms and edible fruit plants. Species of *Citrus* are of great economic significance in many countries of the world and there is still ample scope for the production of new varieties through the use of chemical mutagens on ovular and seedling calli.

Fruit tissue cultures such as that of the pericarp of peanut (RANGASWAMY *et al.*, 1965), mesocarp of apples (LETHAM, 1958), banana (MOHAN RAM and

STEWARD, 1964; TONGDEE and BOON-LONG, 1973), avocado (SCHROEDER *et al.*, 1962; BLUMENFELD and GAZIT, 1971), lemon (KORDAN, 1967) etc. have failed to respond organogenetically beyond rooting, with the exception of tomato (NYSTERAKIS, 1961) and squash (SCHROEDER, 1968). Neither organogenesis nor embryogenesis has been reported in callus cultures of grape berries (HAWKER *et al.*, 1973).

Clonal multiplication of cacao, cassava, banana and edible fruit trees such as guava, custard apple, pomegranate, sapota and papaya using tissue culture methods, still remains an unplumbed field for the horticulturalist. Tapioca callus, though successfully established, has failed to regenerate either shoot buds or embryos (PRABHUDESAI and NARAYANASWAMY, 1975).

Members of the family Amaryllidaceae, Iridaceae and Liliaceae (HUSSEY, 1975) often develop plantlets directly on stem explants without being mediated through callus. But species of *Lilium*, *Hippeastrum* and *Amaryllis* have shown high propensity for regeneration in culture. Plants which will respond in tissue culture are often those which regenerate easily in vivo.

Leaf tips of seedlings and mature plants of many orchids readily form calli in which regeneration can be manipulated (CHURCHILL *et al.*, 1973; Chap. I.2,3. of this Vol.). Isolated leaves of many dicotyledonous species are highly regenerative as for instance, members of the Acanthaceae, Begoniaceae, Scrophulariaceae and Solanaceae. Leaf mesophyll cells of tobacco have been demonstrated to be totipotent (JULLIEN, 1971).

Attempts have been made to establish tissue cultures of the various bromeliads but with only partial success (MAPES, 1973). Protocorm-like bodies and embryos have been observed to develop in culture but these have failed to differentiate further. Species of cactus and the succulents have received little attention although valued as ornamental plants.

Many members of the Compositae, Leguminoesae, Solanaceae and Orchidaceae respond to apical meristem culture which finds its widest application in obtaining pathogen and virus-free plants. Regenerants arising from proliferating callus cells of shoot tip segments have all been diploid (GAMBORG *et al.*, 1974) as in many legumes, and may be a means of obtaining genetically uniform plants.

Propagation of tree genera such as *Ilex* by conventional methods of breeding is time-consuming and often inefficient owing to low seed-set, poor germination and great variability. Cell culture techniques could be applied in order to overcome these barriers and may prove to be a short cut in reducing the normal life cycle which may take several months. Somatic callus induction has been accomplished with sufficient numbers of tree species (WINTON, 1972a, b, 1974a, b). However, regeneration of whole plants has been realized in only a few and is often limited to the formation of a shoot or a root. In forest tree propagation, uniformity of types and phenotypically desirable individuals is an important consideration (see Chap. I.5. of this Vol.). Also, the production of plantlets via embryogenesis rather than shoot buds is desirable as this would ensure the endowment of a tap root, so essential for anchoring the plant deep into the soil. Conifer tissue cultures have not responded to whole plant morphogenesis (DURZAN and STEWARD, 1970) except in *Pinus palustris* where complete plantlets have been obtained (SOMMER *et al.*, 1975). Experimental morphogenesis aimed at the

development of vegetative propagation methods, of the important timber species of Black Cherry (CAPONETTI *et al.*, 1971) and teak (MEHRA, personal communication) through tissue culture is underway. A beginning has been made in the application of tissue and cell culture technique for coconut palm (BLAKE *et al.*, 1974), date palm (REUVENI *et al.*, 1972), and oil palm (STARITZKY, 1970b), ordinarily lack vegetative means of propagation.

Loss of capacity for organogenesis attributed to age, senescence, nutrition and ploidy changes are considered the main obstacles in the way of exploiting tissue culture as a tool for practical plant propagation, but these can only alter the degree of regeneration. It would be best therefore to avoid the use of calli that have been continuously subcultured and to use only the primary callus which often retains competency for morphogenesis. In exceptional instances, however, leaf tissue cultures such as that of tomato that had undergone 30 passages in culture, were still found to retain their organogenetic capacity for propagation on a massive scale (PADMANABHAN *et al.*, 1974).

Freeze-preservation of cell cultures (see Chap. VII.3. of this Vol.) may be resorted to in rare plant species. An interesting observation has been that callus of seedling or of embryo origin are potentially more regenerative than calli derived from adult phases of plant growth.

Clonal tissue from single cell isolates from the pith of diploid tobacco var. Wisconsin 38 has yielded offspring that were all tetraploid (MURASHIGE and NAKANO, 1966) and so may prove of advantage in obtaining polyploid plants and in the possibility of introducing a new variety using tissue culture as a potential tool. Production of polyploid plants from tissue cultures has been realized in *Brassica oleracea* (HORÁK *et al.*, 1971).

In any scheme of plant improvement, tissue and cell culture techniques could serve as a supplement to the conventional methods of plant breeding and as a means towards production of plants with novel features. Chemical mutagens and ionizing radiations can be used in bringing about genetic diversity in plants regenerated from somatically mutant cell types.

Morphogenetic expression in a particular direction is manifested as a result of the cumulative effect of the subtle interactions and balances among the growth-regulating substances and nutrient constituents mediated through various physical factors. Physical factors such as temperature, humidity, photoperiod, pressure, magnetic fields etc. are important morphogenetic tools, as they control the internal milieu of the cell but have not been fully exploited.

Protoplasts isolated enzymatically from various tissues could form cell clusters which eventually regenerate into plants (see Chap. IV.1. of this Vol.). Production of new genotypes in commercially important species by experimental modification of cells through protoplast fusion between unrelated genomes and transformation of cells by uptake of foreign DNA in order to recover viable plants as a routine, suggest immense potential in the realm of cell and tissue culture of protoplast and cell culture for plant improvement (BAJAJ, 1974; CARLSON and POLACCO, 1975).

References see page 207.

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Chapter II Haploids

1. Anther Culture: Haploid Production and Its Significance

J. REINERT and Y. P. S. BAJAJ

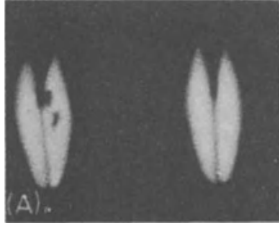
1. Introduction

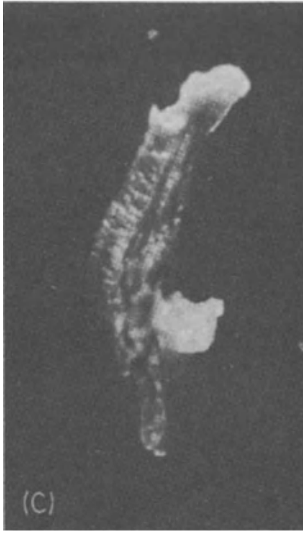
Haploid plants have the gametophytic number of chromosomes, that is a single set of chromosomes in the sporophyte. Their significance for plant improvement, and as a tool in various disciplines of plant science has been stressed (see KASHA, 1974a). Since haploids are of great importance, especially in studies on the induction of mutations and also for the production of homozygous plants, they are needed in large numbers. However, the conventional methods (KIMBER and RILEY, 1963; MAGOON and KHANNA, 1963), employed by plant breeders for their production are cumbersome, laborious and not very efficient. With the introduction of techniques for the induction of androgenesis by the culture of excised anthers (GUHA and MAHESHWARI, 1964), isolated pollen (NITSCH, 1974a) and by chromosome elimination through hybrid embryo culture (KASHA and KAO, 1970) it has become increasingly evident that tissue culture methods could considerably speed up the production of haploids for breeding programs. These topics are dealt with in detail in the subsequent articles in this chapter.

In 1953, TULECKE, for the first time, observed that mature pollen grains of a gymnosperm *Ginkgo biloba* can be induced to proliferate in culture to form haploid callus. This and subsequent work (TULECKE, 1959; TULECKE and SEHGAL, 1963) established that pollen grains, under in vitro conditions, can be manipulated to bypass their primary role of forming sperms, and instead can be induced to form haploid tissue. It was not until 1964 that GUHA and MAHESHWARI reported the direct development of embryos from microspores of *Datura innoxia* by the culture of excised anthers. Later BOURGIN and NITSCH (1967), obtained complete haploid plants of *Nicotiana tabacum*. Since then, there has been a steadily increasing stream of information. In this article, which is intended to serve as a general introduction to the chapter on haploids, the methods of anther culture and the significance of haploids for plant improvement are discussed.

2. Techniques

The technique for the excision and culture of anthers is relatively simple and efficient. Closed flower buds, which have anthers (Fig. 1 A) containing uninucleate microspores (Fig. 2A), are most suitable for the induction of androgenesis. The flower buds are excised, surface-sterilized in batches of 25 with a 5% solution of commercially available bleach or a 1% calcium hypochlorite solution for 10 min,


(A)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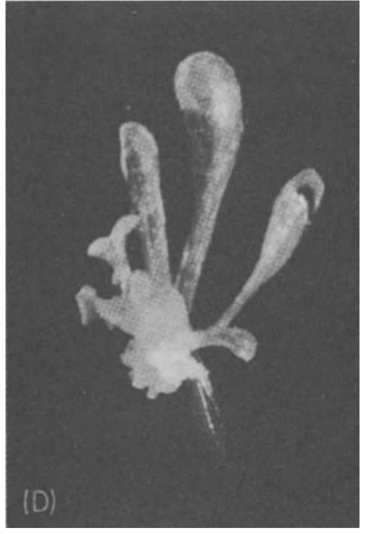
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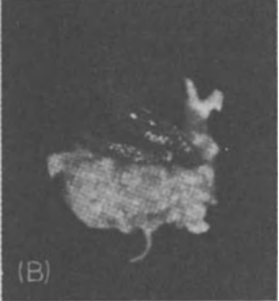
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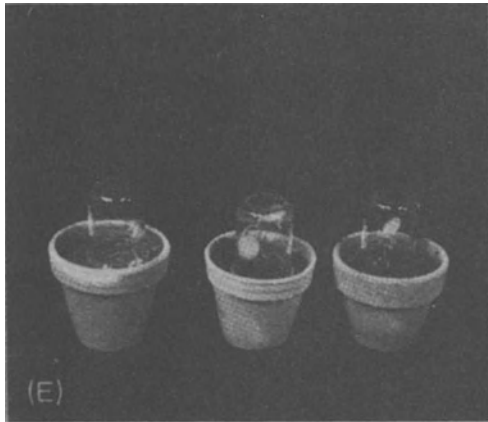
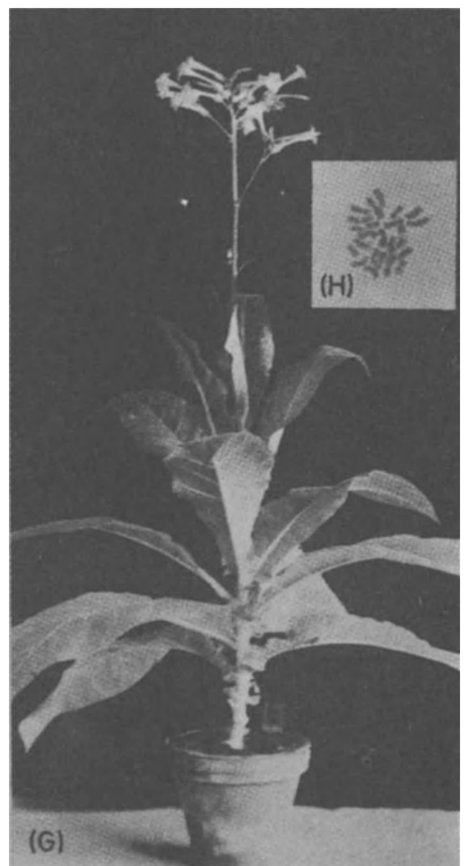

(E)


(H)
(G)

(F)

and then washed twice with sterile, distilled water. However, the flower buds obtained from plants grown in green houses, if dissected carefully, need no sterilization. First an incision is made on one side of the flower bud and the stamens are gently taken out with a pair of fine forceps and collected in a sterile petri dish. The filament from the stamen is then carefully removed and five anthers are normally transferred to each culture vessel. During excision of anthers special care should be taken to ensure that they are not injured in any way. Damaged anthers should be discarded, as they often tend to produce callus from parts other than pollen. The anthers are cultured on agar-solidified medium in glass tubes or small petri dishes. They can also be grown in liquid media in Erlenmeyer flasks and kept on a slow rotary shaker. The cultures are incubated at 24–27° C and exposed to light of about 2000 lux for a 14 h day. Although unopened flowers with their sepals and petals touching each other have been observed to be suitable for androgenesis by excised anther culture, this is only true of young plants. However, older plants, especially towards the end of flowering, frequently produce small buds which, although appearing to be at a suitable stage, contain anthers with a heterogeneous mixture of microspores and young pollen. In such anthers the number of abnormal pollen also increases and androgenesis is not only delayed but considerably reduced. Therefore, it is important that anthers should be taken from relatively young plants which have been grown under good light conditions (DUNWELL and PERRY, 1973).

Depending upon the plant species it takes about 3–8 weeks for pollen plantlets to emerge from the anthers (Fig. 1 B). The plantlets (Fig. 1 D) when approximately 5 cm tall can be removed from the culture and freed from agar by gently washing with running tap water. The plantlets are then transferred to small pots containing autoclaved soil. To reduce sudden shock and to prevent dessication, it is advisable to cover these plantlets with glass beakers (Fig. 1E), and to keep them in a well-lit, humid green-house. After one week the beakers are removed, and after a further two weeks, when the potted plantlets have grown to a sufficient size (Fig. 1F) they are again transferred into larger pots (Fig. 1G) where they eventually develop to maturity and flower.

Haploids can be diploidized to produce homozygous plants by two methods; (1) Colchicine treatment: the plantlets while still attached to the anther are treated for 24–48 h with 0.5% colchicine solution (KASPERBAUER and COLLINS, 1972; BURK *et al.*, 1972), washed thoroughly and replanted. However, if mature haploid plants are available then colchicine-lanolin paste (0.4%) may be applied to the axils of the leaves (TANAKA and NAKATA, 1969). (2) Stem-segment culture: it is known that haploid callus cultures frequently undergo endomitosis to form

Fig. 1A–H. Culture of excised anthers and the regeneration of haploid plants (A) Tobacco anther at the time of inoculation (containing uninucleate microspores). (B, D) Anthers 4 and 6 weeks after culture; note the emergence of embryos and plantlets from the bursting anther. (C) 5-Week-old potato (*Solanum tuberosum*) anther undergoing proliferation to form callus. (E) 6-week-old haploid tobacco plants transferred from culture tube to pots. To prevent dessication, the plants are kept in high humidity under glass beakers for a couple of days. (F) Same, 2 weeks after trasnfer to pots; note the unfolding of the leaves. (G) A haploid plant showing flowering 8 weeks after transfer to soil (total time after culture of anther is 14 weeks). (H) A chromosome squash from root tip of a tobacco plant showing haploid number ($n=24$)

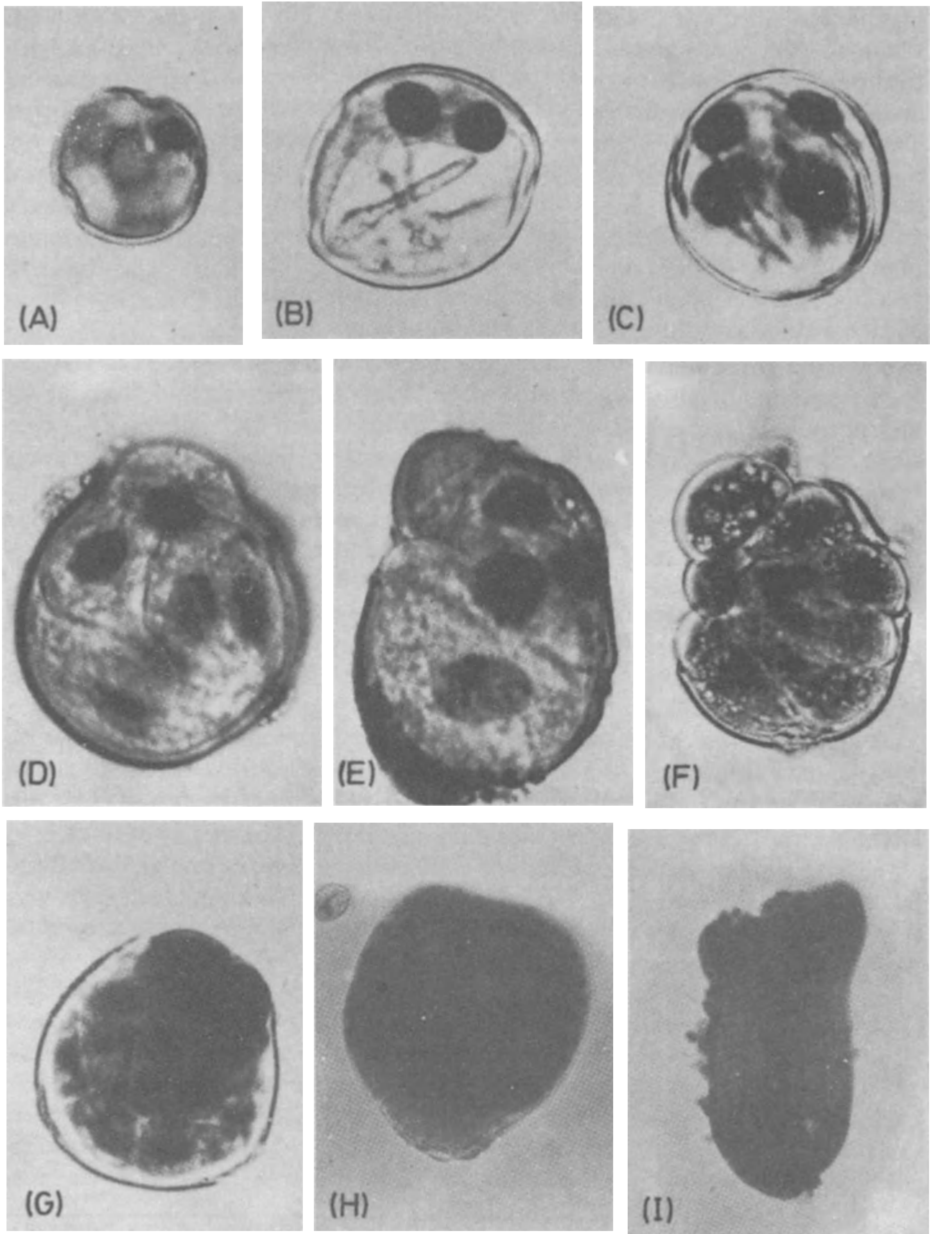


Fig. 2A–I. In vitro induction of androgenesis from microspores of *Nicotiana tabacum*. (A) Uninucleate microspore in an anther at the time of inoculation. (B) Binucleate microspore showing two similar nuclei taken from an anther grown for 4 days, after cold treatment. (C) Four nucleate pollen grain taken from 10-day-old culture. (D–G) 2 and 3-weeks old microspores in cultures showing early stages of androgenesis. (H, I) Heart and torpedo-shaped haploid embryos from a 4-week-old culture. (From REINERT *et al.*, 1975)

diploid cells and this property can be exploited to obtain homozygous diploids (NITSCH, 1969; KAMEYA and HINATA, 1970; KOCHHAR *et al.*, 1971). In such experiments a small segment of stem is grown on an auxin-cytokinin medium to induce callus formation (NITSCH, 1972). During callus growth diploid homozygous cells are produced by endomitosis from which a large number of isogenic diploid plants can be differentiated.

3. Culture Media and Nutritional Requirements

Basal media (see Table 1) of WHITE (1943), MURASHIGE and SKOOG (1962), and NITSCH and NITSCH (1969), with slight modifications and the addition of growth regulators, have been used for the culture of excised anthers. The normal level of sucrose is 2–4%, however, barley (CLAPHAM, 1971), tomato (SHARP *et al.*, 1971a), and wheat (OUYANG *et al.*, 1973) anthers have been observed to grow better on media with 6–12% sucrose; this seems to be an osmotic effect rather than a need for a higher carbohydrate level.

The iron in the medium plays a very important role and is indispensable. Although androgenesis can be initiated in tobacco without any iron, the proembryos do not develop beyond the globular stage. Chelating agents such as FeEDTA (NITSCH, 1969) and FeEDDHA (RASHID and STREET, 1973) are more efficient than ferric citrate as the source of iron.

The nutritional requirements of the excised anthers are much simpler than those of isolated microspores. In the isolated microspores it is obvious that certain factors responsible for the induction of androgenesis, which might have been provided by the anther, are missing, and these have to be provided through the medium. This is certainly the case in tobacco, where excised anthers can be cultured successfully on a simple basal medium, while the isolated microspores require higher amounts of nitrogen in the form of amino acids (NITSCH, 1974a; REINERT *et al.*, 1975).

In plants such as tobacco (NITSCH, 1969) and *Atropa* (RASHID and STREET, 1973), complete androgenesis can be realized on simple media, but in most cases (see Table 2) a certain balance of auxin/cytokinins or complex additives such as casein hydrolysate, yeast extract, coconut milk and other extracts have to be incorporated into the medium. These observations support the assumption that the requirement for auxin and cytokinin depends on their endogenous level in the anther.

Media rich in growth regulators encourage the proliferation of tissues other than microspores (i.e. anther wall, connective and filament) and should be avoided, because in such cases mixed calli with cells of different ploidy levels are obtained (NISHI and MITSUOKA, 1969; DEVREUX *et al.*, 1971; NARAYANASWAMY and CHANDY, 1971; ENGVILD *et al.*, 1972; IYER and RAINA, 1972; SUNDERLAND *et al.*, 1974; WAGNER and HESS, 1974; Chap. III.1 of this Vol.). This would necessitate a laborious screening program for the selection of haploid cells and plants.

Incorporation of activated charcoal into the medium has stimulated the induction of androgenesis in tobacco anthers (ANAGNOSTAKIS, 1974). Our work

Table 1. Composition of some of the basal media commonly used for the culture of excised anthers

	WHITE (1943)	MURASHIGE and SKOOG (1962)	NITSCH and NITSCH (1969)
Macronutrients		mg/l	
$Ca(NO_3)_2 \cdot 4H_2O$	288	—	—
KNO_3	80	1900	950
NH_4NO_3	—	1650	720
KCl	65	—	—
KH_2PO_4	—	170	68
$NaH_2PO_4 \cdot 4H_2O$	19	—	—
$CaCl_2 \cdot 2H_2O$	—	440	166
$MgSO_4 \cdot 7H_2O$	737	370	185
Na_2SO_4	200	—	—
Micronutrients			
$Fe_2(SO_4)_3$	2.5	—	—
$FeSO_4 \cdot 7H_2O$	—	27.8	27.8
Na_2-EDTA	—	37.3	37.3
$MnSO_4 \cdot 4H_2O$	6.7	22.3	25
H_3BO_3	1.5	6.2	10
$ZnSO_4 \cdot 4H_2O$	2.2	8.6	10
KI	0.75	0.83	—
$Na_2MoO_4 \cdot 2H_2O$	—	0.25	0.25
$CuSO_4 \cdot 5H_2O$	—	0.025	0.025
$CoCl_2 \cdot 6H_2O$	—	0.025	—
Organic			
Biotin	—	—	0.05
Glycine	3.0	2.0	2
Inositol	—	100	100
Nicotinic acid	0.5	0.5	5
Pyridoxine-HCl	0.1	0.5	0.5
Thiamine-HCl	0.1	0.1	0.5
Folic acid	—	—	5
Sucrose	20000	30000	20000

(BAJAJ *et al.*, 1976) with *Nicotiana tabacum* cv. Badischer-Burley has shown that the percentage of androgenic anthers can be raised from 41 to 91 by the addition of activated charcoal (2%) to the medium (Fig. 3), however, it seems that charcoal can also cause small increases in the induction of diploid plants (Table 3). Although no satisfactory explanation can be given at present for the stimulatory effect of charcoal, it seems likely that it might be responsible for removal of inhibitory substances from the medium. In this connection FRIDBORG and ERIKSSON (1975) have observed that charcoal enhances the induction of embryogenesis in cell suspensions of carrot, where embryo formation cannot normally be induced by the omission of auxin from the medium (see REINERT, 1968). Likewise they observed abundant root formation in old cultures of *Allium cepa* when transferred to charcoal medium; such cultures do not normally produce roots. This led them to conclude that charcoal might be removing auxin from the

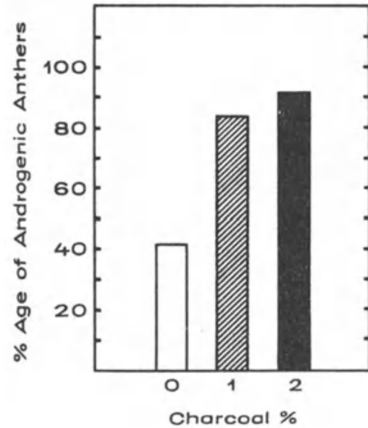


Fig. 3. Effect of various concentrations of charcoal on androgenesis in tobacco anthers grown for 12 weeks. (After BAJAJ *et al.*, 1976)

medium. Although charcoal has highly increased the efficiency of adrogenesis in tobacco anthers it does not seem to be so much effective for the anthers of *Atropa belladonna* and *Petunia hybrida* (BAJAJ, unpublished). It is possible that in plants like tobacco charcoal absorbs inhibitory substances and thus reduces the number of potential pollen embryos which would normally have aborted. However, it seems more likely that the level of growth regulators, both endogenous and exogenous, is regulated by absorption onto charcoal.

4. Induction of Androgenesis

In culture, microspores undergo various modes of androgenesis (Fig. 4) which lead to the formation of haploids either directly by embryogenesis, or indirectly via callus formation (Fig. 5). From the geneticist's point of view the direct formation of embryos from pollen is preferable to the indirect formation of embryos via callus. Haploid plants or tissues have been obtained from species of a number of families (for literature see Chap. III.1). These are *Aegilops caudata, Arabidopsis thaliana, Asparagus officinalis, Atropa belladonna, Brassica oleracea, Capsicum annuum, Coffea arabica, Datura innoxia, D. metel, D. meteloides, D. muricata, D. wrightii, Fragaria virginiana, Hordeum vulgare, Lolium multiflorum, Lotus corniculatus, Lycium halimifolium, Lycopersicon esculentum, Nicotiana alata, N. attenuata, N. glutinosa, N. knightiana, N. otophora, N. raimondii, N. rustica, N. sylvestris, N. tabacum, Oryza sativa, Pelargonium hortorum, Petunia auxillaris, P. hybrida, Secale cereale, Setaria italica, Solanum dulcamara, S. melongena, S. nigrum, S. tuberosum, S. verrucosum, Triticale and Triticum aestivum.*

In addition, in this dynamic field androgenesis has recently been induced in *Agropyron repens* (ZENKTELER *et al.*, 1975), *Brassica napus* (THOMAS and WENZEL, 1975a), *Bromis inermis* (ZENKTELER *et al.*, 1975), *Digitalis purpurea* (CORDUAN and SPIX, 1975), *Festuca pratensis* (ZENKTELER *et al.*, 1975), *Freesia* (BAJAJ and PIERIK, 1974), *Hyoscyamus niger* (CORDUAN, 1975; RAGHAVAN, 1975), *Paeonia hybrida* (SUNDERLAND, 1974), *P. lutea* (ZENKTELER *et al.*, 1975) *Pharbitis nil* (SANG-

Table 2. Some plant species[a] of economic and horticultural importance in which haploid plants/calli have been obtained by the culture of excised anther/pollen

Plant species	Family	Inoculum	Medium[b] (mg/l)	Growth response	Reference[c]
Asparagus officinalis	Liliaceae	anther	mod. MS + NAA (0.1) + BA (0.2)	Callusing and plants	PELLETIER *et al.* (1972), RAQUIN (1973), HONDELMANN and WILBERG (1973)
Brassica oleracea × *B. alboglabra*	Cruciferae	pollen	NM + CM (10%)	Callusing	KAMEYA and HINATA (1970)
B. napus	—	anther	LS + 2,4-D (0.1) + CM (10%)	Callusing and embryos	THOMAS and WENZEL (1975a)
Capsicum annum	Solanaceae	anther	MS + kin(1) + 2,4-D(1) + RNA-nucleotide (40)	Callusing and embryos	GEORGE and NARAYANASWAMY (1973), WANG *et al.* (1973a)
Coffea arabica	Rubiaceae	anther	mod.L.S. + 2,4-D(0.1) + kin (0.1)	Callusing and embryos	SHARP *et al.* (1973)
Fragaria virginiana	Rosaceae	anther	mod. MS + NAA(2) + kin (5)	Callusing and plants	ROSATI *et al.* (1975)
Freesia sps	Iridaceae	anther	MS + NAA(0.5) + kin(1) + PBA(2) + CH(500)	Callusing and plants	BAJAJ and PIERIK (1974)
Hordeum vulgare	Gramineae	anther	WM + NAA(1) + CM(15%)	Callusing and plants	CLAPHAM (1971), MALEPSZY and GRUNEWALD (1974)
Lilium longilflorum	Liliaceae	anther	mod MS + thiamin HCl(4)	Callusing	SHARP *et al.* (1971b)
Lotus corniculatus	—	anther	mod LS + IAA(0.1) + kin(0.1)	Callusing	NIIZEKI and GRANT (1971)
Lycopersicon esculentum	Solanaceae	anther	mod.MS + NAA(2) + kin(1)	Callusing and plants	GRESSHOFF and DOY (1972)
L. esculentum	—	pollen	WM + 2,4-D(6)	Callusing	SHARP *et al.* (1971a)
L. esculentum	—	pollen	N and N + IAA(0.1) + anther extract	Callus, embryos	DEBERGH and NITSCH (1973)
Nicotiana tabacum	—	anther	N and N	Embryos and plants	BOURGIN and NITSCH (1967), NAKATA and TANAKA (1968), NITSCH and NITSCH (1969), SUNDERLAND and WICKS (1969, 1971)
N. tabacum	—	pollen	N and N + glutamine (800) + serine (100) + IAA(0.1) + zeatin (0.01) + inositol (500)	Embryos and plants	NITSCH (1974a, b), REINERT *et al.* (1975), BAJAJ *et al.* (1975)
Oryza sativa	Gramineae	anther	MS + NAA (1)	Callus, embryos and plants	NIIZEKI and OONO (1968, 1971), NISHI and MITSUOKA (1969), HARN (1969, 1970), GUHA *et al.* (1970), GUHA-MUKHERJEE (1973)

Table 2. (continued)

Plant species	Family	Inoculum	Medium[b] (mg/l)	Growth response	Reference[c]
Paeonia hybrida	Ranunculaceae	anther	MS+NOA (0.1)	Embryos and plants	SUNDERLAND (1974)
P. lutea	—	anther	MS+IAA(1)+kin(1)+CH(500)	Embryos	ZENKTELER *et al.* (1975)
Pelargonium hortorum	Geraniaceae	anther	mod MS+NAA(2)+ kin(2.5)+CM (15%)	Callus and plants	ABO-EL-NIL and HILDEBRANDT (1971)
Petunia hybrida	Solanaceae	anther	mod MS+BA(0.2)+NAA(0.1)	Callus, embryos and plants	BERNHARD (1971), RAQUIN and PILET (1972)
Populus sp.	Salicaceae	anther	—	Callusing	SATO (1974)
Primula obconica	Primulaceae	anther	MS+2,4-D(0.5)+Zeatin (2)	Callusing and plants	BAJAJ (1976)
Saintpaulia ionantha	Gesneriaceae	anther	Blaydes medium (1966)	Plants	HUGHES *et al.* (1975)
Secale cereale	Gramineae	anther	MS+2,4-D(0.25)	Callus, embryos and plants	THOMAS and WENZEL (1975b)
Solanum melongena	Solanaceae	anther	B and N+CM(15%)	Callusing and plants	RAINA and IYER (1973)
S. tuberosum	—	anther	MS+NAA (0.01)	Embryos and plants	DUNWELL and SUNDERLAND (1973)
Triticale	Gramineae	anther	MS+2,4-D (2)	Callusing	WANG *et al.* (1973b)
Triticum aestivum	—	anther	MS+2,4-D (2)	Callus, embryos and plants	OUYANG *et al.* (1973), PICARD (1973), PICARD and DE BUYSER (1973), WANG *et al.* (1973a), BAJAJ (1976)
Vitis vinifera	Vitaceae	anther	mod. MS+NAA (0.1)+kin (0.3)	Callusing	GRESSHOFF and DOY (1974)
Zea mays	Gramineae	anther	WM+2,4-D(2)	Callusing and roots	MURAKAMI *et al.* (1972)
Z. mays	—	pollen	NM+serine(100)+glutamine (800)	Embryos	NITSCH (Chap. II. 2)

[a] For ploidy level of the plants regenerated by the anther culture refer to Chap. III.1.
[b] Different media have been used by various workers for the same plant species, however, in the table only the simple medium is given.
[c] For detailed references see Chap. III.1 and page 331.

Abbreviations: Basal media: B and N: BOURGIN and NITSCH (1967), MS: MURASHIGE and SKOOG (1962), LS: LINSMAIR and SKOOG (1965), N and N: NITSCH and NITSCH (1969), NM: NITSCH (1974a), WM: WHITE (1943).
Supplements: IAA: Indoleacetic acid, NAA: Naphthyleneacetic acid, NOA: Naphthoxyacetic acid, CH: Casein hydrolysate, CM: Coconut milk, kin: kinetin, BA: Benzylamino purine, PBA: 6-(benzylamino)-9-(2-Tetrahydropyranyl-9H-purine, 2,4-D: Dichlorophenoxyacetic acid.

Table 3. The effect of charcoal (2%) on the percentage of androgenic tobacco anthers and on the ploidy of regenerated plants (The results are based on 500 plants)

	Control	Charcoal medium
	Percentage	
Androgenic anthers	41	93
Haploids	96.5	95
Diploids	3.5	5

WAN and NORREEL, 1975), *Populus* (SATO, 1974), *Primula obconica* (BAJAJ, 1976), *Saintpaulia ionantha* (HUGHES *et al.*, 1975) and *Vitis vinifera* (GRESSHOFF and DOY, 1974).

5. Ontogeny of Androgenesis

In vivo, as a result of meiosis in pollen mother cells, pollen tetrads are formed, which are eventually released in the form of microspores. The newly formed microsspore is highly cytoplasmic with a central nucleus. With the increase in volume of the microspore and vacuolation, the nucleus is pushed towards the periphery. By the first mitosis, a large and diffuse vegetative cell and a small dense generative cell are formed. The former remains quiescent while the latter divides to form sperms (Fig. 4A).

In culture, although androgenesis can be induced in anthers at the tetrad stage (GRESSHOFF and Doy 1972b), or at the binucleate pollen stage (KAMEYA and HINATA, 1970), however, microspores just before, or at the time of first mitosis, are most suitable for the induction of androgenesis (NITSCH and NITSCH, 1969). Microspores in cultured anthers exhibit various modes of development (SUNDERLAND, 1974) leading to androgenesis. Some notable ones represented in Figure 4, are; 1. After first mitosis the vegetative cell divides repeatedly to form a haploid embryo (Fig. 4B) as is the case in *Nicotiana* (SUNDERLAND and WICKS, 1971), *Datura* (IYER and RAINA, 1972), and *Hordeum* (CLAPHAM, 1971). 2. The generative nucleus usually remains quiescent or aborts after a few divisions, but, occasionally it (Fig. 4C) does take part in androgenesis (DEVREUX *et al.*, 1971). 3. The microspore nucleus instead of dividing to form a generative and a vegetative nucleus, gives rise directly to two similar nuclei (NITSCH, 1974a) or there is a direct segmentation (RASHID and STREET, 1973, 1974) where both the daughter cells are involved in androgenesis (Fig. 4D). 4. In some cases, as in *Datura* (SUNDERLAND *et al.*, 1974) two similar nuclei (Fig. 2B) formed as a result of direct division of the microspore nucleus or of the vegetative one, fuse with one another (Fig. 4E) and this results in the formation of homozygous diploids.

Irrespective of the early events in the division of the microspore nucleus there are two modes of androgenesis, the direct and the indirect, which are diagrammatically represented in Figure 5. 1. *Direct androgenesis*: in this type the microspore behaves like a zygote and undergoes various stages of embryogeny simulating

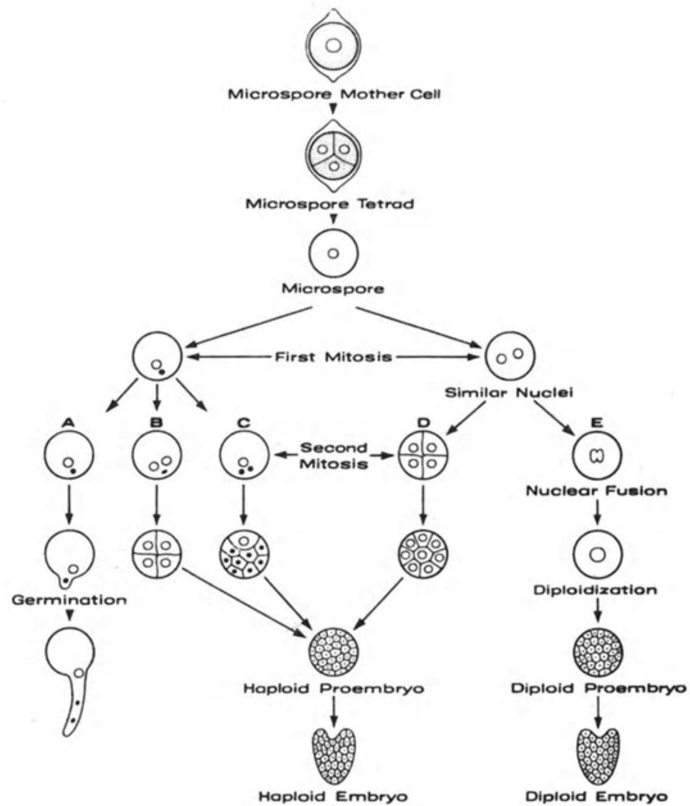


Fig. 4 A-E. Diagrammatic illustration of microsporogenesis, and various modes of development of pollen under in vivo and in vitro conditions. (A) Normal second mitosis of pollen in vivo forming two sperms and the germination of pollen to form a pollen tube. (B–E) In vitro behavior of pollen. (B) Repeated division of the vegetative nucleus and the abortion of the generative nucleus. (C) Formation of a haploid embryo as a result of repeated division of the generative nucleus, while the vegetative nucleus aborts. (D) Haploid embryo formation as a result of repeated division of two similar nuclei of a pollen. (E) Homozygous diploid embryo formed by fusion of two similar nuclei of the pollen after first mitosis. (Modified from DEVREUX, 1970)

those in vivo, as in *Atropa*, *Datura* and *Nicotiana*. The embryos, mostly at the globular stage, are released from the exine (Fig. 2G) and develop further (Fig. 2H, I). Finally the cotyledons unfod and the plantlets emerge from the anthers (Fig. 1 B, D) in 4–8 weeks. 2. *Indirect androgenesis*: in contrast to the direct androgenesis, the microspores instead of undergoing embryogenesis, divide a few times to form a callus which bursts through the anther wall (Fig. 2D–F). This mode of development is quite common (see Table 2) and is usually caused by complex media, and in cases where the polarity seems to be disturbed. The callus either differentiates to form embryos, or roots and shoots on the same medium, or it has to be transferred to another medium. The callus-derived plants are mostly undesirable as they exhibit genetic variations and polysomy. For details on the ploidy status of anther-derived plants see Chapter III.1.

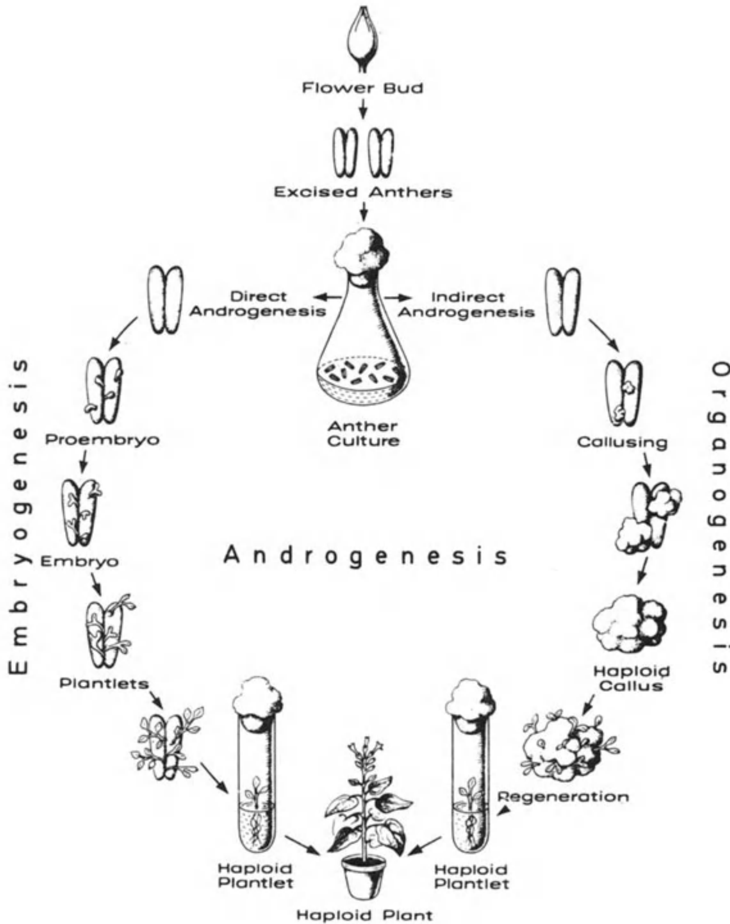


Fig. 5. Schematic representation of the culture of excised anthers and the development of haploid plants directly by embryo formation, or through haploid callus

6. Stability of Haploid Cell Cultures

One of the main problems with tissue cultures, especially those of haploid origin, is the maintenance of their genetic stability in long term cultures (D'AMATO 1975; Chap. III.1). For their use in somatic cell genetics, it is essential that the cultures remain haploid during the early stages when the mutants are being selected. The possibility of using para-fluorophenylalanine (PFP) to stabilize or even enhance the growth of haploid cultures has been explored by various workers, however, there is considerable disparity in the results. GUPTA and CARLSON (1972) observed that PFP (9 mg/l) inhibited the growth of diploid tobacco (*Nicotiana tabacum* cv. Havana Wisconsin 38) callus of stem origin, whereas the growth of haploid cells remained uneffected. Likewise, DIX and STREET (1974) observed growth inhibition

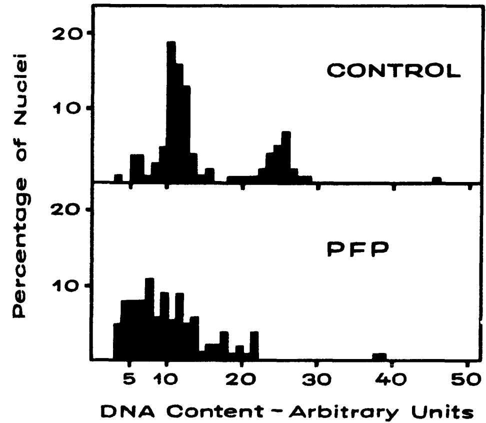


Fig. 6. Effect of 10 mg/l of para fluorophenylalanine (PFP) on frequency distribution of values for the nuclear DNA contents of cell suspension of halploid *Nicotiana tabacum* cv. Badischer-Burley grown for 4 weeks. DNA content was estimated by microspectrophotometry of 100 Feulgen-stained nuclei. The PFP treated cultures show an increase in the number of haploid cells with corresponding decrease in the diploid cells. (From BAJAJ and GRÖBLER, unpublished)

Table 4. Effect of various concentrations of PFP on the growth (percentage of control) of haploid stem-explants of *Nicotiana tabacum* cv. Badischer Burley cultured for 6 weeks. (From BAJAJ and GRÖBLER, unpublished)

Weeks after culture	PFP (mg/l)					
	5		10		15	
	Hapl.	Dipl.	Hapl.	Dipl.	Hapl.	Dipl.
1	140	130	150	110	160	130
3	280	130	250	70	340	110
6	150	140	210	140	190	100

of diploid cultures of *Nicotiana sylvestris* by PFP (37.5 mg/l), but did not observe any preferential growth of haploid cells in mixed cultures, and stated that it is not the ploidy level, but the genotype, that determines the sensitivity to PFP. Recently, MATTHEWS and VASIL (1976) reported that PFP inhibits the growth of both haploid and diploid pith-explants of *Nicotiana tabacum*, and suggested that PFP acts as a selective inhibitor which reduces the growth rate of haploid cells less than that of the cells of higher ploidy.

In our experiments (BAJAJ and GRÖBLER, unpublished) with *Nicotiana tabacum* cy. Badischer-Burley stem explants, the optimal effect of PFP was observed 3 weeks after the initiation of the cultures (Table 4), and 15 mg/l was found to be more effective than 5 or 10 mg/l both for the haploid, as well as for diploid cultures. Whereas 15 mg/l of PFP was stimulatory for the haploid tissues, it slightly inhibited the growth of diploid explants. Almost similar results are obtained with *Atropa belladonna.*

In cell suspensions obtained from androgenic plants, initially about 70% of the cells were haploids, but, within 4–6 weeks the level decreased to about 30%. After eight months of repeated subculturing, only 5% of the cells showed the haploid DNA level. PFP enhanced the maintenance of such cultures for up to four

weeks (Fig. 6). The behavior of such cells in long-term PFP cultures is under investigation.

The disparity in the results obtained by various workers could be due to the differential sensitivity of various genotypes to PFP. Like DIX and STREET (1974), we also observed that results varied a great deal with different plants. The physiological state and the age of the plant appear to be of utmost importance, and in order to critically evaluate the effect of PFP on growth, especially of the stem-explants, it might be necessary to grow the haploid and diploid plants under the same conditions. In addition our microspectrophotometric data clearly reveal that PFP at 10 mg/l helps to maintain the haploidy in short-term cell suspension cultures. This is an area which needs to be further explored as it might prove to be helpful in preventing polysomy in haploid cultures, or in understanding the mechanism underlying the increase in ploidy.

7. Significance and Uses of Haploids

Haploids are required for two main reasons, (1) The presence of one set of chromosomes facilitates the isolation of mutants, and (2) isogenic diploids can be obtained by chromosome diploidization. Their significance for plant improvement has been appreciated (see KIMBER and RILEY, 1963; MAGOON and KHANNA, 1963). Although, by conventional inbreeding and backcrossing, it is possible to obtain pure lines, this is a time-consuming process. With the availability of the in vitro methods there has been a renewed interest in the production of haploids (see DEVREUX, 1970; SUNDERLAND, 1970; MELCHERS, 1972; NITSCH, 1972; PANDEY, 1973; KASHA, 1974a; BAJAJ, 1976). By anther culture, haploids can be obtained in a matter of weeks, and by doubling their chromosome number homozygous diploids can be procured in a single generation. These fertile homozygous plants can be used for producing the inbred lines required to utilize hybrid vigor. Such pollen-derived homozygous diploid plants offer the advantages that they show normal meiotic segregation (COLLINS and SADASAVAIAH, 1972) and that they do not lose desirable characters by segregation.

With a view to inducing mutations, NITSCH *et al.* (1969) subjected young tobacco plants (while still emerging from the anthers) to 1500 and 3000 rads of gamma irradiations and obtained a high proportion of abnormalities in their size, shape and color. In a later study NITSCH (1972) reported that white-flowered tobacco mutants developed when anthers were grown on a medium supplemented with N-3-nitrophenyl-N-phenylurea. Likewise, X-ray-subjected anthers produced 40% haploid plants with aberrent phenotypes, and about 6% with chromosomal aberration (DEVREUX and SACCARDO, 1971).

Haploid cell cultures are also useful material for the study of somatic cell genetics (SMITH, 1974), especially for mutation and cell modification (see Chap. IV.3). By employing agar-plating techniques and suspension cultures, isolated protoplasts, pollen and cells (Fig. 7) can be handled like microbes, and can be treated with various mutagenic chemicals and irradiations. The advantage of the haploid cells is that the mutants can be easily diploidized to form homozygous

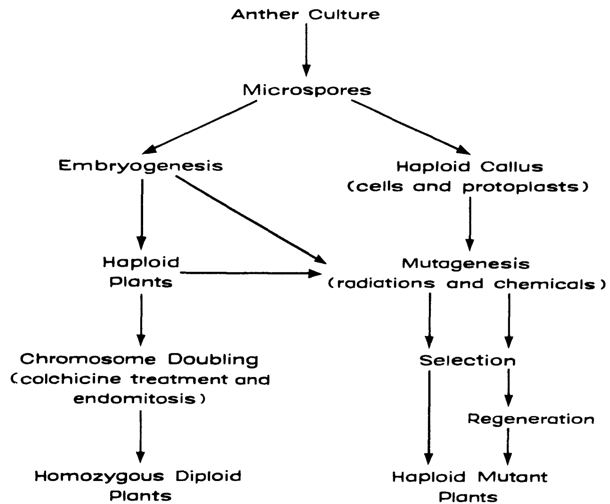


Fig. 7. Schematic representation for the induction of homozygous diploids and mutants from haploid plants, cells and protoplasts obtained through anther culture

diploids. In this connection isolated pollen grains (NITSCH, 1974a; REINERT *et al.*, 1975) and possibly their protoplasts (COCKING, 1973; BAJAJ, 1974, 1975) would be better suited for this purpose.

TULECKE (1960) isolated arginine-requiring strains of cells from *Ginkgo* pollen by substituting auxin in the medium with L-arginine. Later, CARLSON (1970) obtained auxotrophic mutant cell lines by treating haploid tobacco cells with ethyl methane sulfonate, and regenerated plants from such cells. Since then, mutant cell lines have been obtained by other workers by treating *Nicotiana tabacum* and *Petunia hybrida* cells with 5-bromodeoxyuridine (MALIGA *et al.*, 1973a), streptomycin (BINDING *et al.*, 1970; MALIGA *et al.*, 1973b) and DNA base analogs (LESCURE, 1973). By subjecting haploid *Nicotiana tabacum* cells to methionine sulfoximine CARLSON (1973) also claimed to have regenerated mutant plants which showed a considerably lower level of infection when inoculated with wildfire pathogen, *Pseudomonas tabaci.*

Since haploid plants can be regenerated from the isolated mesophyll protoplasts of tobacco (OHYAMA and NITSCH, 1972; BAJAJ, 1972), *Petunia (*BINDING, 1974) and *Datura* (SCHIEDER, 1975) they should form an additional tool for studying the effect of various mutagens. Mesophyll protoplasts have the advantages of being relatively homogenous, divide more synchronously and are genetically more stable than the callus cultures.

Employing haploid tomato (*Lycopersicon esculentum*) cell cultures DOY *et al.* (1972, 1973a,b) reported their transformation by the incorporation of phage lambda (Bacterial virus). However, the observation that these cells grow on a medium containing galactose and lactose for some time, is not sufficient proof for their transformation. This work has obvious shortcomings but could be extended to other systems in order to find out the feasibility of employing haploid cells for transformation studies. More conclusive results could be expected using proto-

plasts instead of callus cells because of the possibility of a better uptake of the transforming factor.

The importance of haploids in cereal breeding has been dealt with in detail in article 3 and 4 of this chapter. To summarize, in addition to other advantages, haploids can be used to obtain homozygosity for genes in cases where it is normally difficult to achieve, such as self-incompatible alleles in rye. In barley, monoploids provide a means of gamete selection, and if they are produced from F_1 hybrids then the number of generations of self pollination, that are normally required to produce uniform lines, are eliminated. This saving of the number of generations is very important in winter barley because of the time required for vernalization. A second advantage of doubled monoploids is the reliability of the selection provided. When screening for yield, quality or disease resistance one can be sure that due to homozygosity the desirable characters will not be lost because of segregation from heterozygous loci in later generations (see JENSEN, 1974a). Of the various other uses of cereal haploids, the most remarkable one is the one-step transfer of cytoplasm from one line to another (CHASE, 1952). Using *Zea mays* monoploids GOODSELL (1961) and KERMICLE (1973) successfully transferred the genotypes of inbred lines into cytoplasm that caused male sterility. In addition they could be used in (1) recurrent selection, (2) fixing quantitative characters so that they can be analyzed and selected and (3) the identification of superior crosses for more extensive exploitations (Chap. II.4).

Potato *(Solanum tuberosum)* haploids have been sought after for years (LAMM, 1938) because of their potential in breeding programs (HOUGAS and PELOQUIN, 1958). However, the methods employed for their production are laborious and involve wide crosses. By anther culture (DUNWELL and SUNDERLAND, 1973) haploids and subsequently the homozygous diploids can be obtained in a relatively short time. The fertile dihaploids obtained from tetraploids can be used for breeding at the diploid level.

Haploids will be of immense importance for the improvement of some crop plants. For instance, the haploids of complex hybrids of *Coffea arabica* (see Chap. I.6) are of special interest in relation to 'coffee rust disease' because such hybrids result from the incorporation of resistance genes of wild coffee after artificial pollination. The objective of work with these crosses is to combine multiple desirable genes in a single genotype. Six known dominant genes confer rust resistance, and 1/64 of the microspores in F_1 from a hexahybrid cross will carry all such genes, providing there are no linkages. Furthermore, the resulting totally homozygous diploids will have a double dose of the genes for resistance and will have an advantage over their parent lines because of the phenomenon of gene dosage. When a haploid line of a disease-resistant heterozygous wild species, having one or more genes for rust resistance is used, the product following diploidization should have a higher potential in field utilization, if there is a gene dosage effect.

In some plants of horticultural importance, such as *Freesia* (BAJAJ and PIERIK, 1974) which are normally propagated vegetatively by means of corms, it takes 8–10 years to produce a clone which is large enough for commercial purposes. In these cases anther culture has obvious advantages. Specific desirable

gene combinations may be linked in a haploid form and then converted to the fertile homozygous diploid or polyploid forms in a relatively short time.

Another use of haploids is in relation to self-incompatible plants. The haploids from self-incompatible plants would be highly desirable as they would have several applications, some of which outlined by de NETTANCOURT and DEVREUX (Chap. III.6), are (1) the use of completely homozygous self-incompatible diploid lines as parental material for the production of F_1 hybrid seed, and (2) for the production of haploid plants from interspecific hybrids between self-compatible and self-incompatible species. Such plants would theoretically be genetically different from one another and would carry different combinations of paternal and maternal chromosomes. After diploidization they should constitute an extensive collection of substitution lines.

In addition they could be used in (1) recurrent selection, (2) fixing quantitative characters so that they can be analyzed and selected, (3) the identification of superior crosses for more extensive exploitations (Chap. II.4).

Amongst other uses, homozygous plants, obtained through anther culture, have also been employed for the selection of breeding lines of *N. tabacum* with high alkaloid content (COLLINS *et al.*, 1974). Haploid protoplasts can also be used as a means of clonal propagation of rare haploids, and in cases where the frequency of haploid induction by anther culture is low.

However, it must be pointed out that there are some drawbacks in the technique of anther culture. One is that the plants may not originate from pollen only but also from various other parts of the anther. This results in plants with various levels of ploidy (NISHI and MITSUOKA, 1969; DEVREUX *et al.*, 1971; NARAYANASWAMY and CHANDY, 1971; ENGVILD *et al.*, 1972; IYER and RAINA, 1972; SUNDERLAND *et al.*, 1974; WAGNER and HESS, 1974). The ploidy status of these plants has been dealt with in detail in Chapter III.1, and it is evident that an extensive screening and selection of such anther-derived plants is required. However, this can be partially overcome by the culture of isolated pollen (Chap. II.2). There is, of course, the possibility of fusion of pollen nuclei, but this would be an advantage, because homozygous cells would be directly obtained without any application of colchicine.

References see page 331.

2. Culture of Isolated Microspores

C. NITSCH

1. Introduction

In 1953 Tulecke succeeded in growing a callus from the pollen of *Ginkgo biloba* when it was cultured in vitro. The callus had the haploid number of chromosomes, however it has not been possible to regenerate plants from such callus. After the discovery by GUHA and MAHESHWARI (1964) that *Datura* anthers placed in culture stimulated the pollen to produce embryos, and the production of haploid plants from anther culture of *Nicotiana* by BOURGIN and NITSCH (1967) opened a new line of research which is especially attractive for geneticists and plant breeders. This discovery would be even more significant if the knowledge already available for single cells in vitro could be applied. In 1970 KAMEYA and HINATA using more or less the same culture medium as that used in anther culture observed the formation of cell clusters in mature pollen grains of *Brassica.* These pollen grains were released from pre-sterilized anthers and cultured in drops of between 50 and 80 in slide glass holes. These authors however did not show that the cell clusters observed were derived from single grains, so the possibility of agglomerate of pollen grains is not excluded. Later, SHARP *et al.* (1972) with *Lycopersicon esculentum*, and PELLETIER (1973) with *Nicotiana tabacum* stimulated colony formation by the nurse-culture technique, however it is only in the case of *Nicotiana* that whole plant could be obtained using *Petunia* callus tissue for nursing. The following pages are therefore devoted to a description of the isolation of the pollen and its cultural conditions (NITSCH, 1974a) a technique which has been successfully applied to *N. tabacum* cv. Badischer Burley (REINERT *et al.*, 1975; BAJAJ *et al.*, 1976). As will be shown, this involves two different steps, each of equal importance. First, the induction of the microspore to change its normal sexual evolution and follow a vegetative pathway; second, the development of the induced microspore towards embryogenesis.

2. Induction towards a Non-Sexual Pathway

From the work done on anther culture, successful for a fairly large number of species (see REINERT and BAJAJ, Chap. II.1), the authors are unanimous in saying that the state of the pollen at the initiation of culture is critical. In order to produce haploid tissue, anthers should be planted at the time of the first pollen mitosis or very soon after.

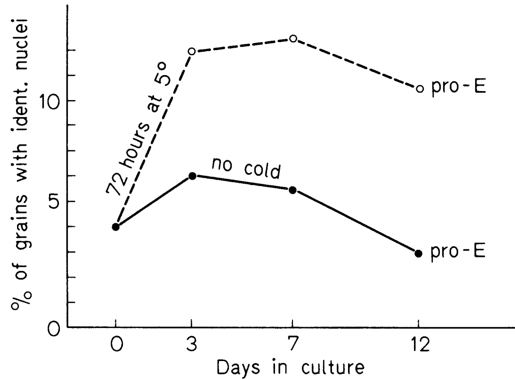


Fig. 1. Effect of cold shock on the pollen nuclei. Flower buds of *N. tabacum* placed at 5° C for 3 days before taking out the anthers *(upper curve)*, and anther planted just after excising the bud from the plant *(lower curve)*. In both cases, microspores isolated from the anther 3 days after placing the anther in culture. 1000 microspores stained by Feulgen's method are observed for each point. Pro-embryos at day 12 have 4 identical nuclei

By modifying their normal development, we hope to trigger isolated microspores into behaving as somatic cells which would give rise to plantlets. The idea was therefore to prevent the microspore from forming a generative nucleus, thinking that, liberated from the influence of the generative cell, the vegetative cell could grow as any somatic cell. Any treatment which can modify the mitosis has therefore been studied.

1. Following the work of SAX in 1937 on *Tradescantia*, NITSCH and NORREEL in 1972 carried out some experiments in which plants underwent a cold shock at the time of the first pollen mitosis. A sudden change in temperature i.e. from 20° C down to 3 or 5° C respectively for *Datura* or *Nicotiana* was given either to the whole plant, to the flower-bud, to the dissected anther or to the isolated microspores either in the light or in the dark.

The effect on the microspore nuclei was analyzed by observing grains which had been fixed for 2–3 days in 1:3 v/v acetic acid: ethanol and stained in Schiff's reagent after 10 min hydrolysis in 60° C 1 N HCl.

The greatest effect is obtained when the cold treatment is given to the flower bud detached from the plant and kept in the dark in a humid environment. The percentage of pollen in which the mitosis has been modified (i.e. containing two identical nuclei instead of one vegetative and one generative) is higher after a cold shock, as is shown in Figure 1.

2. It is known from the literature that low temperature breaks down microtubules. As was suggested by JENSEN (1974b) this effect could be either replaced or increased by gravity. Indeed, for *Nicotiana sylvestris* where it is difficult to trigger the development of the microspore in culture, gravity increases the number of haploid plants produced by pollen culture.

The flower buds are centrifuged down at 500 g in a 5° C refrigerated centrifuge for 1 h before anthers are dissected out.

The same type of response has been obtained with *Zea mays* although these results are still preliminary and unpublished.

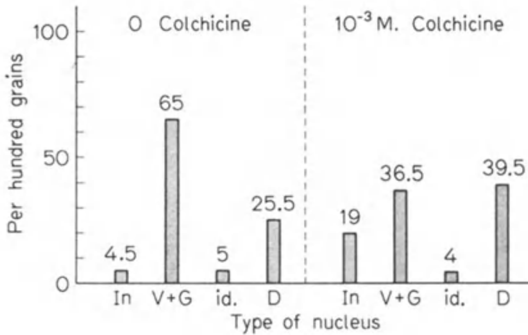


Fig. 2. Effect of colchicine on the first pollen mitosis of *N. tabacum*. Nuclei of 1000 grains stained with Feulgen are observed 3 days after the colchicine treatment. *1n* grains with one nucleus, *V+G:* grains with one vegetative and one generative nucleus, *id:* grains with 2 identical nuclei, *D:* dead grains

3. Certain chemicals are also known to have an effect on mitosis, namely the growth substances. As shown by DEBERGH and NITSCH in 1973 for tomato, the first divisions towards embryogenesis seem to be auxin-dependent. However, the monocotyledons appear to be more generally auxin-dependent. NIIZEKI and OONO (1968) with rice, PICARD and DE BUYSER (1973), and OUYANG *et al.* (1973) with wheat, indicate the absolute requirement for auxin in the medium for the production of haploid plants by anther culture of these species. As we shall see later in the discussion on the nutritional requirements for microspore culture, we believe that growth substances have to be used with great care in order to avoid dedifferentiation of the tissue.

4. Another way to induce microspores to grow as somatic cells is by altering their mitoses with the use of colchicine. This chemical is known (JENSEN, 1974b) to stop mitosis at metaphase by inactivation of the spindle mechanism: weak doses of colchicine are used for doubling the chromosome number of a cell. The action of colchicine on the microspore could therefore be particularly advantageous, since it would change the microspore into a homozygous diploid cell. The plants originating from such cells would have the great advantage of being fertile unlike the normal haploid plants. Experiments done with *N. tabacum* cv. Red Flowered and Coulo produced 15–20% fertile diploid plants. This percentage is even higher with *N. sylvestris*.

Experimental procedure: anthers of *N. tabacum* or *N. sylvestris* are floated under vacuum in basal medium containing 0.05% colchicine with 2% dimethylsulfoxide, used as a carrier, for 4–12 h in the dark. The anthers are then taken out of the colchicine medium, washed, and placed in fresh liquid medium for three days (inductive period) in a culture chamber at 25–27° C and a light intensity of 3000 lux.

The microspores are then isolated from the anthers and placed in culture as described below. The observations made on the nuclei at the start of the culture are shown in Figure 2. After the colchicine treatment, the number of uninucleate grains increased from 4.5–19%; it is also interesting to note that these uninucleate grains have a much larger nucleus then the normal microspore nucleus. As might be expected the number of dead grains has also increased. Another advantage of

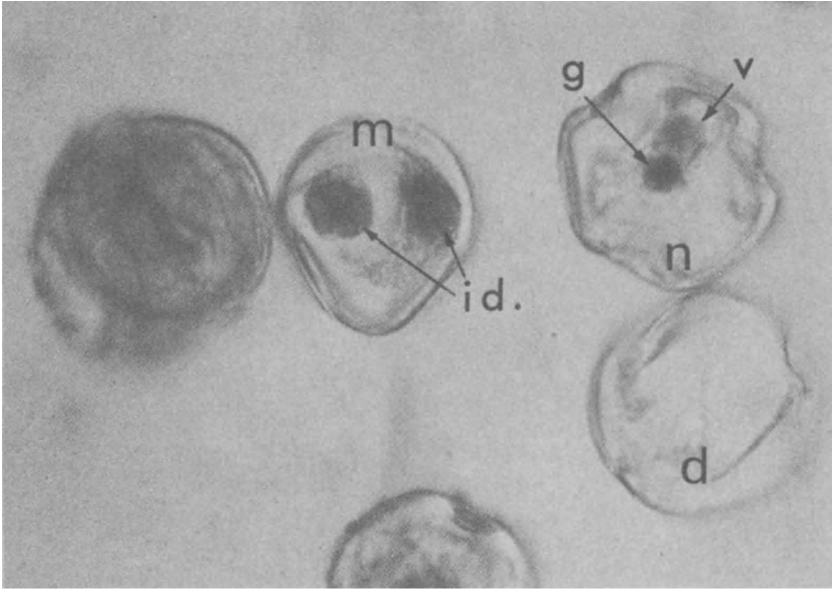


Fig. 3. Induced microspores of *N. tabacum* after 5days in culture (× 1200). *m*: grains in which the mitosis has been modified: 2 identical nuclei, *n*: grains with normal mitosis: *v*: vegetative nucleus, *g*: generative nucleus, *d*: dead grain *id*: grains with 2 identical nuclei

doubling the chromosome number of the microspore might be the production of homozygous diploid in species in which the haploid plants could not survive.

The important factor in obtaining the maximum number of microspores which develop along the vegetative pathway, is the use of anthers which contain the maximum possible number of grains which are in mitosis to start the induction.

The inductive period is rather short (from two to five days) but nevertheless essential, and has to be adapted to each species. It is by staining the microspores following Feulgen's method and observing the presence of grains with two identical nuclei that one can be sure of a high induction frequency (Fig. 3). Thus having observed grains with two identical nuclei, one can go on to culture the isolated microspores by placing them on an appropriate medium which will be described later.

3. Technique for the Isolation of Microspores

The unopened buds are sterilized by a 2 min immersion in a freshly prepared filtered aqueous solution 7% of calcium hypochlorite followed by rinsing twice with sterile water.

When the species requires only an induction by physical shock given to the whole bud, as in *Datura*, the anthers are dissected and pollen extracted immedi-

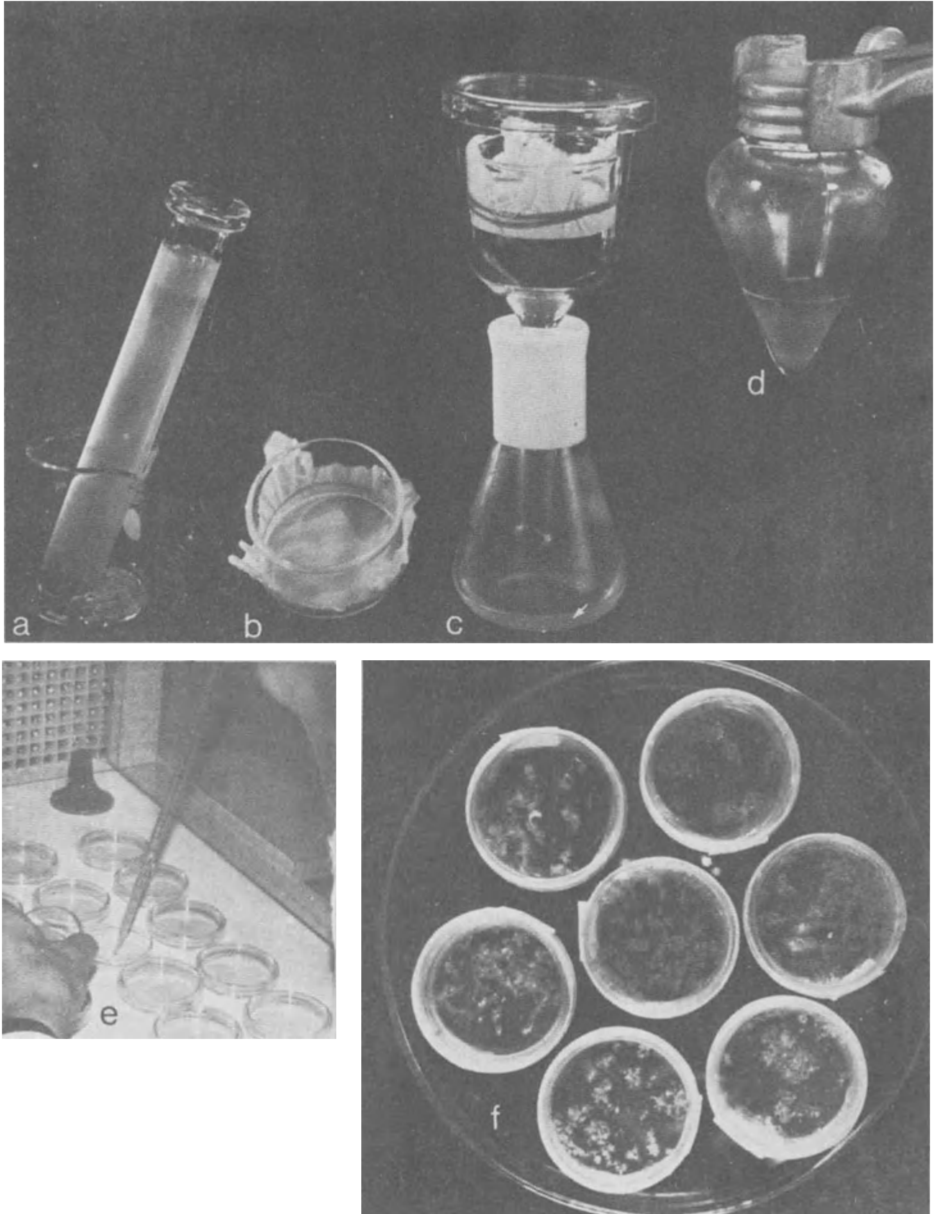


Fig. 4. Technique for microspore culture (a) anther or small flower buds are squeezed against the side of the beaker to push out the pollen into the basal medium. (b) nylon sieve held onto pyrex cylinder by a rubber band. (c) filtering anther mixture through the sieve. The arrow shows the pollen suspension obtained. (d) pollen, centrifuged down, is washed twice with fresh medium. (e) clean pollen suspension distributed into 5 cm pyrex dish: 2.5 ml per dish at 10^3 grains per ml. (f) 14 petri dishes sealed and placed together in a larger dish. Plantlets are seen in the dish after 3 weeks

ately. On the other hand if the species requires an extra inductive period in the presence of chemicals, the anthers are floated on the inductive medium in liquid culture for two or three days. This is so in *Nicotiana* as well as cereals. In short the microspores are extracted from the anthers at the end of the inductive period when microspores with two identical nuclei can be seen (Fig. 3).

In the case of large anthers, as in *Nicotiana*, about 50 anthers are placed in a small beaker containing 20 ml of basal medium (see Fig. 4a). The pollen grains are then squeezed out of the anthers by pressing them against the side of the beaker with the piston of a syringe. Anther tissue debris is removed by filtering the suspension thus obtained through a nylon sieve (Fig. 4b) having a pore diameter which is slightly wider than the diameter of the pollen (e.g. 40 microns for *Nicotiana* or *Datura*, 25 microns for tomato, 100 microns for maize etc.). This pollen suspension (Fig. 4c) is then centrifuged down at low speed (500–800 revolution min for 5 min) the supernatant containing fine debris is discarded and the pellet of pollen resuspended in fresh medium and washed twice (Fig. 4d). The rinsings are particularly important if the anther tissue contains inhibitory substances which would prevent any growth of the microspores. At this point the pollen is mixed with an appropriate culture medium at a density of 10^3 to 10^4 grains per ml. The final suspension is pipetted into 5 cm wide pyrex petri dishes at 2.5 ml per dish (Fig. 4e). To ensure good aeration, the layer of liquid in the dish should be as thin as possible. Each dish is then sealed with parafilm to avoid dehydration, and 14 dishes are placed together in a 20 cm-wide dish (Fig. 4f). These are incubated at 27–30° C for *Nicotiana*. In species in which the flower buds are very small, as for example with fruit trees or cereals, it is difficult to remove the anthers. In these cases we separated each floret from the main axis, cutting off the bud at the level of the receptacle and treated the whole bud in the same manner as for the anthers of *Nicotiana*. A pollen suspension can be just as easily prepared using these whole buds as by using only anthers.

The microspores are best grown in liquid medium, but if necessary can also be grown by plating in a very soft agar medium (0.6% agar) Here again the layer of medium in the dish should be very thin.

4. Requirements for the Growth of the Embryo

4.1 Nutrient Requirement

After numerous experiments in anther culture, a few authors came to the conclusion that some species need only a rather simple medium to produce haploid plants (J.P. Nitsch,, 1969 for *Nicotiana*, Dunwell and Sunderland, 1973 for potato). In *Nicotiana* and *Datura* Nitsch showed that pro-embryos can be produced on 2% sucrose in distilled water. That is to say that mineral salts, minor elements, vitamins and growth substances, all constituents generally used in tissue culture, are not necessary for the first phase of development of the microspores. To grow these pro-embryos to complete plants, the same author showed that only iron plus sucrose were necessary. This work gave the first indication that the

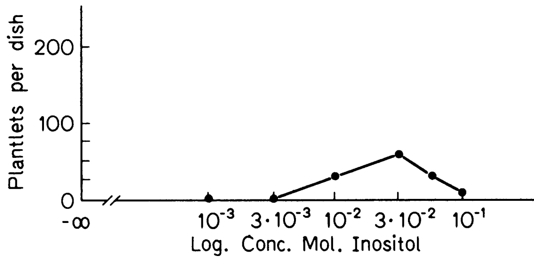


Fig. 5. Effect of myo-inositol on the growth of androgenetic embryos of *N. tabacum*. Induced microspores are placed on basal medium supplemented with myo-inositol at different concentrations. Average number of plantlets from 6 dishes after one month in culture

production of haploid plants from anther culture was a two-step procedure, namely induction and nutrition, and led us to believe that part of the nutritional requirement came from the surrounding tissue.

4.1.1 Effect of Sugar

In anther culture, sucrose was shown to be absolutely necessary for most species and was not successfully replaced by other sugars. The influence of glucose shown by HOMES (1967) on the development of somatic embryos of carrot was found to be negative for induced microspores of Solanaceae. On the other hand the positive effect of a high concentration of myo-inositol shown by NORREEL and NITSCH (1968) on the production of somatic embryos in carrot tissue could be reproduced for *Nicotiana* microspores. Figure 5 indicates the effect of different concentrations of myo-inositol added to the basal medium. A concentration of 0.5% allows the development of an average of 58 plants per dish, that is around 0.5% of the total number of pollen in culture.

4.1.2 Effect of Amino Acids

Amino acids have long been known to be necessary for the development of zygotic embryos. In 1953 PARIS *et al.* studied the effect of aspartic acid and glutamic acid and their amides on the development of zygotic embryos of *Datura*. Glutamine was found to increase the growth of these embryos. In our experiments, glutamine added to the basal medium allowed the development of microspores isolated from the anther as shown in Figure 6. A concentration of 3×10^{-3} M on an average produces plants from around 2% of the grains per dish. Asparagine, lysine and threonine did not give any growth. Hydroxyproline, on the other hand, in preliminary experiments produces plantlets at a concentration of 3×10^{-4} M. The partial success of nurse culture technique for the production of haploid plants from pollen by SHARP *et al.* in 1972 with tomato, and PELLETIER, 1973 for *Nicotiana* suggest that the microspore benefits for its development from nutrients originating from the anther tissue. This hypothesis was confirmed when in 1972 we succeeded in growing microspores of *Datura innoxia* isolated from the anther on medium conditioned by an aqueous extract of embryogenic anther tissue. Further experiments on the analysis of such extracts have shown the importance of another amino acid, namely serine, for the development of the proembryo (NITSCH, 1974a, b). Thus a completely defined medium has been proposed

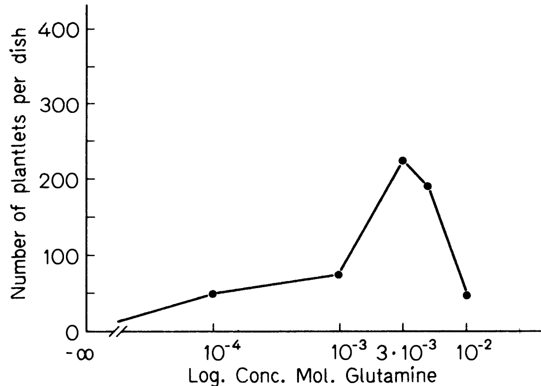


Fig. 6. Effect of L-glutamine on the growth of androgenetic embryos of *N. tabacum*. Induced microspores are placed on basal medium supplemented with L-glutamine at different concentrations. Average number of plantlets from 6 dishes after one month in culture

for the production of haploid plants by in vitro culture of isolated microspores. The basal medium is the classical medium used for anther culture (in mg/l):

Mineral salts: KNO_3 (950), NH_4NO_3 (725), $MgSO_4$, 7 H_2O (185), $CaCl_2$ (166), KH_2PO_4 (68); Iron: 5 ml/l of a solution obtained by dissolving in 1 liter of distilled water 5.57 g of $FeSO_4$, 7 H_2O and 7.45 g of Na_2 EDTA; and: 2% sucrose to which is added myo-inositol at 2.5×10^{-2} M, glutamine at 5×10^{-3} M and serine at 10^{-3} M. The pH is adjusted to 5.8 with NaOH and the sterilization done by filtration.

An average of 5% of the pollen of *Nicotiana tabacum* placed in culture developed to plantlets in this medium. Figure 7 shows the development of *Nicotiana* and corn microspores grown on such complete medium after having been induced towards embryogenesis. For *Nicotiana*, the induction requires two to three days at 5° C for the intact buds followed by three days in the anther on the basal medium in liquid culture prior to the isolation of the microspores. For maize the induction requires low temperature followed by colchicine. The results with *Zea mays* L. are still very preliminary and unpublished.

4.2 Effect of the Environment

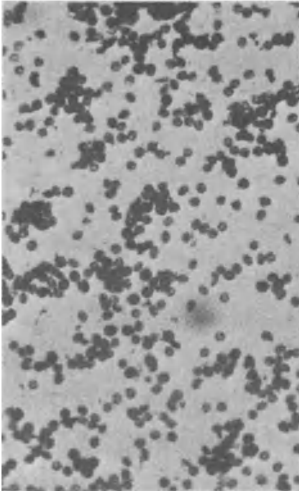
4.2.1 Temperature

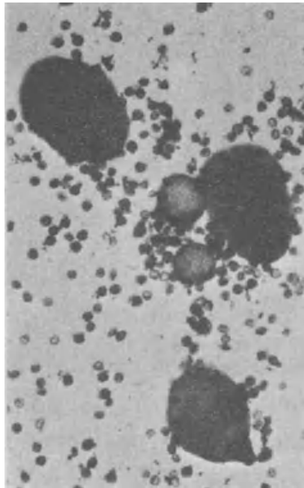
As for any culture, the temperature at which the cells are grown varies for each species, for example, we have shown that *N. tabacum* gives more plants at 27° C than at 22° C. As a rule, one can say that the optimal temperature for pollen development for a species is a few degrees higher than the temperature under which undifferentiated callus tissue grows best.

4.2.2 Light

Plantlets can develop from anther cultures kept in the dark. However, it has been shown that placed in the light, the anthers will develop many more and stronger plants. A series of preliminary experiments done on the effect of light on the growth of plantlets from isolated microspore culture is summarized in Figure 8. As one might expect, microspores lacking protection from the anther tissue are

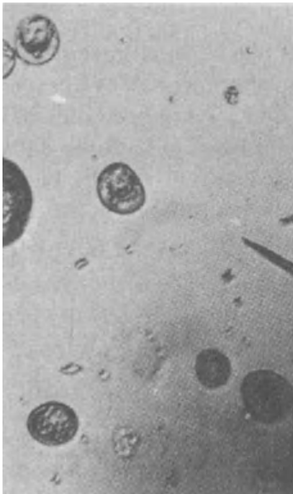
Nicotiana

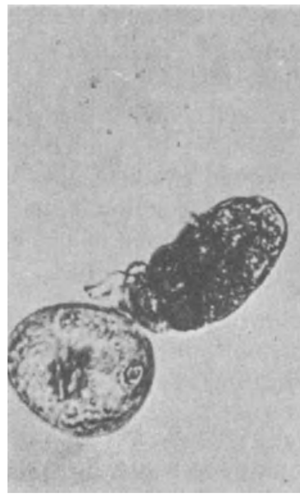


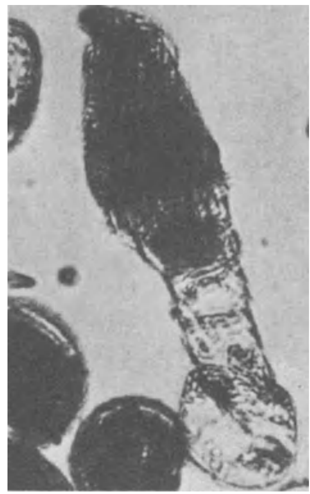


3 days 10 days 21 days

Maize







10 days 21 days 35 days

Fig. 7. In vitro behavior of isolated microspores of tobacco and maize. *Upper Nicotiana tabacum*, 3, 10, and 21 days in culture *Lower Zea mays* L. 10, 21, and 35 days in culture (both in the same complete medium described in the text)

more sensitive to light than when they are grown inside an anther. Red fluorescent light or low intensity white light (500 lux) gave the best results. From these experiments we could also see that the sensitivity of the microspore to light is more important during the first ten days of the culture. Moreover the microspores grown under red fluorescent light developed faster than those under white light.

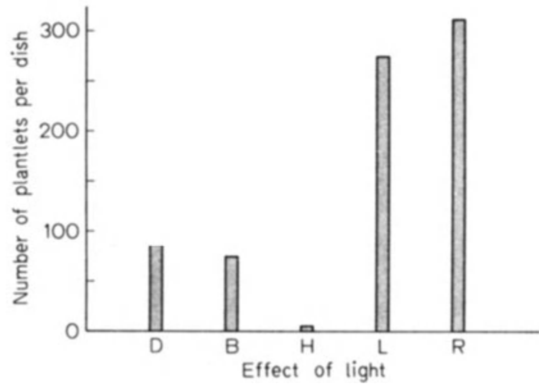


Fig. 8. Effect of various types of light on the growth of *N. tabacum* microspores. Average number of plantlets from 5 dishes after one month in culture. *D* total darkness, *B* blue intensity (500 lux) same as *H*, *R* red fluorescent light

In the red-light-grown culture, the small embryos were already visible with the naked eye after ten days while 15 days were necessary for cultures kept under low-intensity white light.

5. Advantages of Isolated Microspore Culture over Anther Culture

5.1 The Influence of the Anther Tissue Can Be Detrimental

1. Although anther culture of about two dozen species as different as *Nicotiana* and *Oryza* or *Triticum* has been successful in producing haploid plants, the number of plants produced is still very limited and many attempts were unsuccessful. In most cases however, the first divisions of the microspore were observed in culture. This interruption in the development of the microspore may be due to growth-inhibiting substances leaking out of the degenerating anther tissue.

2. In other instances when no inhibition is observed, the diploid tissue, more precisely the connective tissue, is growing very actively. The growth of this diploid tissue is competitive with the growth of the haploid microspore which is very soon submerged by a profuse diploid callus. One commonly reads in the literature that various workers culture anthers on media containing growth substances and leave them for several weeks or even months. In such cases the haploid tissue competes with the other tissue but the presence of growth substances in the medium inhibits the developments of the embryo. The cells originating from the microspore, because of the presence of hormones in the medium, become undifferentiated and grow as a callus. Taking the microspores out of such an environment and placing them on a medium with sugars and amino acids rather than auxins will preserve the embryogenic capacity of the microspore and produce haploid plantlets in large number. On the other hand if one lets the haploid callus grow and then induce it to regenerate plants, it is well known that the ploidy of the plants thus obtained will be very variable and numerous chromosomal alterations and anomalies are generated during culture of undifferentiated tissue (see Chap. III.1).

It is therefore suggested that microspores should be liberated from the anther tissue just after the first divisions are observed. However more work is needed to be able to induce this first division in the isolated cells.

5.2 A Homogeneous Population Will Allow Better Experimentation

1. Unlimited numbers of cells which develop freely into embryos in a liquid medium provide an ideal system for morphological and biochemical analysis. This technique might help us to understand more about the differentation of root and shoot from an isolated cell.

2. Besides the fundamental research which is possible with this new technique of isolated microspore culture, one important field of research is that of genetic and plant breeding. The production of large numbers of haploid plants and homozygous diploids is a big step forward for geneticists.

The isolated microspores mixed at random will give a population more homogeneous than anther culture, moreover the efficiency of mutagenic treatment is greater for single cells than for cells surrounded by another tissue. The techniques used in microbiology for plating as well as for selection of a given character are directly applicable to the microspore culture.

To conclude, it is shown that isolated microspore culture present all the advantages of anther culture plus many more. The number of plants obtained is higher, and the time required to produce them is shorter. It is now possible, at least for *Nicotiana* and *Datura*, to start from a microspore and by doubling the chromosome number of the microspore, collect seeds from the homozygous diploid five months later. This technique, when applied to the cereals and other plants of economic importance, will be an excellent tool for the plant breeders.

References see page 331.

3. Haploid Induction in Cereals

D. H. Clapham

1. Introduction

The first confirmed haploid cereal arose parthenogenetically from *Triticum compactum* after a cross with *Aegilops cylindrica* (Gaines and Aase, 1926). By 1940, the sporadic occurrence of haploid plants, spontaneously or following experimental treatment, had also been described for *Hordeum*, *Oryza*, *Secale* and *Zea* (Kimber and Riley, 1963). Haploids were used by cytologists to reveal the tendencies for chromosomes to pair non-homologously (see Darlington, 1965).

Two encouraging recent developments are the discovery that the cross betwen *Hordeum vulgare* and *H. bulbosum* yields haploid *H. vulgare* progeny (Kasha and Kao, 1970), and that anther culture can yield haploid plants (Guha and Maheshwari, 1964, 1966). Of the many methods that have been proposed for raising haploids of higher plants, anther culture has given most promise of being both effective and of general application. This simple technique was soon extended to rice with some success (Niizeki and Oono, 1968, 1971); immature anthers containing uninucleate microspores were cultured on agar culture medium in test tubes. After about two months, calli appeared from up to 9% of the anthers and sometimes could be differentiated to give haploid plants. Further studies confirmed that the calli developed from the microspores rather than from anther somatic tissue. Barley, wheat, *Triticale* and rye are other major cereals from which haploids have been obtained through anther culture (see Table 1). However at present yields are not high enough.

This article reviews progress in anther culture of cereals, considers its merits and shortcomings in comparison with other methods of raising haploids, and discusses the use of haploids in cereal breeding.

2. Methods of Haploid Induction in Cereals

2.1 Anther Culture

2.1.1 Procedures with Cereal Anther Culture

How the parent plants are grown, from which anthers for culture are to be taken, can strongly influence the yield of plantlets. For example, barley gives better results with plants grown out of doors than in the greenhouse; there are seasonal drifts in yield (Dale and Humphreys, 1973); and results are poor with material grown out of season under lights (see also 2.1.6). The genotype of the parent

Table 1. Summarising the results obtained with anther culture of various cereals

Species	Anther[a] stage	Induction medium: Minerals[c] (and % sucrose)	Induction medium: Growth component (mg/l)	Initial conditions of incubation	% of[b] productive anthers	Embryo or callus	Regeneration medium	Nature of plantlets	Reference
Aegilops caudata × *umbellulata* (No success with 6 other *Aegilops* sp.)	2–3	Miller (3%)	2,4-D 2	25° C, 100 lux	1	C	Omit 2,4-D	Albino, haploid	KIMATO and SAKAMOTO (1972)
Hordeum vulgare cv Sabarlis	2–3	MS (12% or 3%)	e.g. TIBA 0.02 or IAA 1, BAP 1, CM 10% alanine 400	22–27° C, 16 h light 15 W/m²	up to 30 or 11	C	No change	Majority albino, up to 40% green ploidy x, $2x$ or $4x$	CLAPHAM (1971, 1973)
H. vulgare cv Amsel and others	1	MS (12%)	e.g. IAA 1.5, BAP 1.5, 2,4-D 0.5	26° C, 2000 lux	up to 6	C	e.g. MS (sucrose 3%) + IAA 0.2, BAP 0.4, CM 10%	Green and albino, x, $2x$, and polyploid	MALEPSZY and GRUNEWALDT (1974)
Oryza sativa japonica various cvs, also *japonica* × *indica* hybrids	2–3	Miller (3%) or MS (3%)	NAA 1 or 2,4-D 2–10	28° C, dark	up to 9	C	No change or basal + IAA 2, K 4	Green or albino, ploidy x–$5x$	NIIZEKI and OONO (1968, 1971); NISHI and MITSUOKA (1969)
Oryza sativa indica Of 18 cvs, 2 primitive ones from Assam best	2–3	Miller (3%)	CM 15%, YE 1000, IAA 2, 2,4-D 2, K 1 or CM 15%	25° C, dark	up to 26%	E	Omit auxins	Green, haploid	GUHA *et al.* (1970), GUHA-MUKHERJEE (1973)

Table 1. (continued)

Species	Anther[a] stage	Induction medium: Minerals[c] (and % sucrose)	Induction medium: Growth component (mg/l)	Initial conditions of incubation	% of[b] productive anthers	Embryo or callus	Regeneration medium	Nature of plantlets	Reference
Oryza sativa ssp Hsien, Keng and F_1 hybrids	2–3	Miller (6%)	e.g. YE 1000, 2,4-D 2	18–26° C, 9–11 h light 1500 lux	up to 50	C	Basal + IAA 0.2–2, K 0.2–2	6–87% of plantlets albino, rest green. Ploidy *x*–4*x*, nearly 50% diploid	2nd Division (1974)
Secale cereale F_1 hybrid winter types including genes for short culm and self-fertility from *S. vavilovii*	1–5	Nitsch (3%) or MS (3%)	2,4-D 0.1–0.5 or 2,4-D 0.25	25° C, dark	0,14	E or C	No change	Green or albino, ploidy *x*–4*x*	WENZEL and THOMAS (1974), THOMAS and WENZEL (1975b), THOMAS *et al.* (1975)
S. montanum	2–3	MS (12%)	e.g. IAA 0.2, CH 200	24–26° C, dark or light, 1500 lux	1	E or C		Plantlets not obtained	ZENKTELER and MISIURA (1974)
Setaria italica	2–3	Miller (3%)	YE 3000, 2,4-D 1, IAA 1, K 1, or 2,4-D 1, K 2	28° C, dark		C	Change hormones to IAA 2, K 2–4	Green, haploid or diploid	BAN *et al.* (1971)
Triticale	2–3	MS (6%)	e.g. 2,4-D 2, CM 15%	25–30° C, light	up to 17	C	Basal + NAA 0.2, CM 15%	29 albino, 12 green, 1 albino-green, all haploid	WANG *et al.* (1973b)
Triticum aestivum Winter and spring cvs and F_1 hybrids	1–3	MS (3–11%; better at higher concns; usually 6%)	2,4-D 0.7–2, LH 200–300 or (for embryos) CM 30%	15–26° C 9–11 h light 1500 lux	up to 3	C or E	MS (sucrose 3%), IAA 0.2, K 0.2	One third albino, rest green, haploid or occasionally diploid	OUYANG *et al.* (1973)

Table 1. (continued)

Species	Anther[a] stage	Induction medium: Minerals[c] (and % sucrose)	Induction medium: Growth component (mg/l)	Initial conditions of incubation	% of productive anthers[b]	Embryo or callus	Regeneration medium	Nature of plantlets	Reference
T. aestivum Spring wheat hybrids	2–3	MS (6%)	2,4-D 2, K 3 or (for embryos) CM 20%	17–28° C, dark	1	C or E	Basal + NAA 0.2, K 1	9 green, 7 albino, haploid	WANG *et al.* (1973a)
T. aestivum Spring and winter cvs; male-sterile line; F_1 hybrid	4	Miller (12%)	e.g. 2,4-D 0.4	21–23° C, 16 h light	0.5	C	Miller (sucrose 2%) IAA 0.2	Green and albino, haploid	PICARD and DE BUYSER (1973)
T. aegilopoides	1	White (3%)	2,4-D 20	25° C, dark	3	C		Callus only, haploid	FUJII (1970)
T. dicoccoides (No success with *T. monococcum, durum, aestivum* and *speltum*)	1	White (3%)	2,4-D 20	25° C, dark	18	C		Callus only, haploid	FUJII (1970)
Zea mays	2–3	White (2%) or Miller (3%)	e.g. 2,4-D 3–5		0.4	C	e.g. basal + 2,4-D 2	Roots only, haploid?	MURAKAMI *et al.* (1972)

[a] Anther stage, see Table 1.

[b] '% of productive anthers' is a rough guide only. Sometimes the figures apply to short-term experiments with specific varieties, sometimes to average results over a whole season.

[c] MS = minerals after MURASHIGE and SKOOG (1962); Miller = minerals after MILLER (1963); Nitsch = minerals after BOURGIN and NITSCH (1967); White = minerals after WHITE (1943).

CH = casein hydrolysate, CM = coconut milk, LH = lactalbumin hydrolysate, YE = yeast extract, BAP = 6-benzylaminopurine, TIBA = triiodobenzoic acid, K = kinetin.

Table 2. Suggested standard nomenclature for anther staging. (After SUNDERLAND, 1974)

Stage 1: Tetrads or young microspores just released from callose wall. G1 of the cell cycle.

Stage 2: Midphase microspores. Exine well-developed. Vacuole present but nucleus still in G1.

Stage 3: Latephase microspores. Vacuole present. Nucleus in S phase or G2.

Stage 4: First pollen grain mitosis.

Stage 5: Generative and vegetative nuclei present. Generative nucleus cut off by a wall. Microspore vacuole still present.

Table 3. Influence of genotype on the growth response of wheat anthers cultured at uninucleate pollen stage. (After BAJAJ, 1976)

Cultivar	Number of anthers cultured	% of anthers forming callus
1. Maris Ensign	180	—
2. Rothwell Sprite	120	1.6
3. Janus	310	1.6
4. Chinese Spring	250	1.6
5. Kolibri	800	1.7
6. Tilli	421	1.6
7. Cardinal	329	1.5
8. Maris Freeman	180	1.1
9. Maris Widgeon	75	—
10. Maris Huntsman	129	0.8
11. Maris Templar	111	—
12. Maris Dove	85	—
13. Maris Nimrod	167	—
14. Luna	129	—
15. Jubilar 3	151	—
16. Golden Valley	86	—
17. Champlein	141	1.7
18. Bersee	175	0.6
19. Rampton Rivett	172	—
20. 1877	209	—
21. Benno 780	114	—

plants, as well as their physiological status, affects success with anther culture, some varieties being much more responsive than others (see GUHA-MUKHERJEE, 1973).

Inflorescences are removed and the stage of development established by crushing an anther in acetic orcein or carmine and examining the pollen under the microscope. Anthers from stage 1 to stage 5 (Table 2), are recommended for different cereals. The inflorescence is usually surface-sterilized with 5% hypochlorite solution, but one can often omit this and simply remove the anthers aseptically from the florets in a sterile cabinet. The anthers are then placed on an agar culture

medium in tubes or petri dishes, the composition of successful media being outlined in Table 2. With a few exceptions, the concentrated mineral solutions of MURASHIGE and SKOOG (1962), MILLER (1963), and BOURGIN and NITSCH (1967) are preferred to the weaker one of WHITE (1943); an auxin such as 2,4-D, NAA or IAA is almost always included; and sucrose concentrations in the range 6–12% often give higher rates of pollen callus formation than the 2–3% normally recommended for plant tissue culture. Coconut milk, yeast extract, lactalbumin hydrolysate, and various cytokinins and amino acids sometimes help, but no generalization is possible. The requirements for vitamins have not been critically examined.

The cultures are incubated, usually at 25–28° C and initially in darkness. Some but not all rice strains gave higher rates of callus formation at 30° C than at 23–26° C, but the higher temperature was less favorable for subsequent differentiation (2nd Division, 1974). After 3–8 weeks the cultures are checked for anthers forming callus or plantlets. These are put under lights directly or after transference to fresh culture medium to encourage differentiation and further growth.

The media for differentiation or regeneration vary considerably (Table 1). High concentrations of auxins such as 2,4-D are avoided, since they promote callusing rather than differentiation; NAA or IAA and a cytokinin are often added over the range 0.2–2 mg/l; the sucrose concentration is usually 2–3%; natural extracts are sometimes included. Rates of differentiation of calli to give green or albino shoots vary greatly with species and variety, as well as with the composition of the culture medium, from a high of 90% (*Triticale*, WANG *et al.*, 1973b) to zero. Plantlets of rice and barley sometimes regenerate directly from callus on the initial medium in the absence of strong auxins (NIIZEKI and OONO, 1971; CLAPHAM, 1973). Sometimes a plantlet grows directly out of an anther on the initial medium without visible callus formation e.g. with rice (GUHA *et al.*, 1970), wheat (OUYANG *et al.*, 1973; WANG *et al.*, 1973a) and rye (THOMAS and WENZEL, 1975) although it may start callusing later if left on a medium with 2,4-D.

Green plantlets with a developed root system are transferred to compost and grown further, preferably in a mist propagator at first. Then root-tips are taken for chromosome counts. Plantlets of pollen origin from haploid to pentaploid have been observed (Table 2).

It is often desirable to obtain homozygous diploids from the haploids, if doubling has not occurred "spontaneously" in vitro. Colchicine has been used with varying degrees of success (see Chap. II.4; CHASE, 1969 for the doubling of barley and maize haploids respectively). The capping technique of BELL (1950) is of wide application and nearly 100% success with its use has been reported for barley haploids (ISLAM and SPARROW, 1974). The use of the mitotic synchronizer 5-aminouracil before colchicine treatment has been recommended for grass species (see CLARKE, 1969). The origin and significance of diploid plantlets direct from anther culture is discussed in Sections 2.1.2. and 2.1.4. (See also Chapter II.4.).

Haploids set seed in the field after spontaneous doubling at low frequency. In maize about 10% give viable diploid progeny, in barley about 3% (CHASE, 1969; SUBRAHMANYAM and KASHA, 1973). The rate is under genetic control. In rice it can be increased by chronic gamma radiation, which is of value in mutation breeding (TANAKA, 1970).

2.1.2 Pathways to Multicellular Pollen Grains

Anthers can be removed from culture at intervals, fixed, and squashed in chromatin stains and examined (see SUNDERLAND, 1974 for general review; CLAPHAM, 1971 for *Lolium* and barley; IYER and RAINA, 1972 for rice; OUYANG *et al.*, 1973, WANG *et al.*, 1973a; PICARD, 1973 for wheat; SUN *et al.*, 1974a for *Triticale;* WENZEL and THOMAS, 1974, THOMAS and Wenzel, 1975b, for rye. Also relevant,

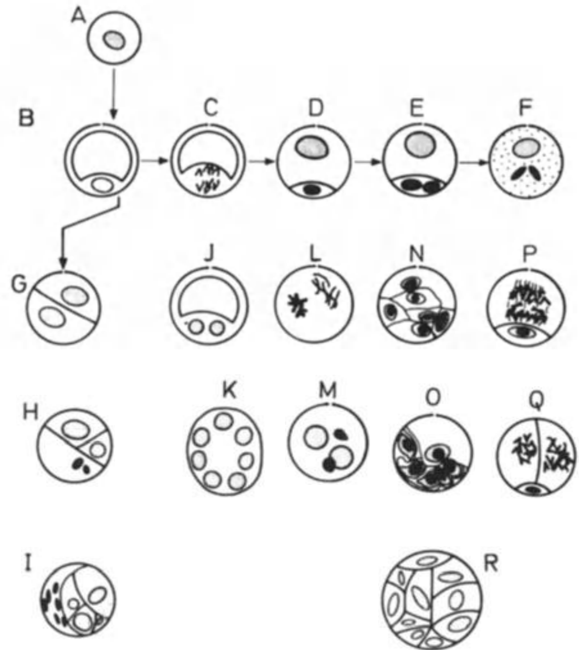


Fig. 1 A–R. Some stages in cereal pollen development, in vivo and in vitro. (A–F) normal development in vivo. Stage numbers after SUNDERLAND'S (1974) standard nomenclature. (A) Young microspore, vacuole undeveloped. Stage 1. (B) Mid- to latephase microspore, vacuole present. Stage 2–3. (C) The normal polarized first pollen mitosis. Stage 4. (D) Young bicellular grain. Generative nucleus against the wall, usually on the opposite side from the germ-pore. Vacuole (not shown) begins to regress. Stage 5. (E) After the second pollen mitosis. Starch grains begin to form. Vacuole (not shown) much reduced. (F) Mature pollen grain packed with starch. Two sperm and a vegetative nucleus, no vacuole. (G) Bicellular grain of wheat in vitro. (After OUYANG *et al.*, 1973). (H, I) *Triticale*, in vitro. See text. (After SUN *et al.*, 1974a). (J, K, L, M) Bi- and multinuclear grains in vitro. Various possible origins, future obscure. In some cases, thin cell wall is perhaps present. (J) Often seen in cereal anther culture. (K) Rye (after WENZEL and THOMAS, 1974). (L, M) *Lolium multiflorum* (after CLAPHAM, 1971). (L) Generative- and vegetative-type nuclei in mitosis. (M) Two generative- and two vegetative-type nuclei. (N, O, P) *Sorghum purpureo-sericeum* (n = 5) with B chromosomes, in vivo. Nucleoli shown, B chromosomes in black (after DARLINGTON and THOMAS, 1941), see also text. (N) 6-celled pollen grain. (O) Vegetative cell and 5 generative cells derived from it; strongly polarized arrangement. (P) Diploid nucleus in anaphase, after nuclear fusion. Lagging B chromosomes. (Q) Rye, in vitro. Two triploid vegetative-type nuclei in mitosis and a generative-type nucleus in interphase. (After THOMAS and WENZEL, 1975b). (R) Multicellular pollen grain of barley. (After CLAPHAM, 1971)

though concerned mainly with anthers in vivo, are DARLINGTON and THOMAS, 1941, on B chromosomes in *Sorghum* and BENNETT and HUGHES, 1972, for effects of ethrel on wheat). It is then seen that some of the microspores develop more or less normally into starch-filled tricellular pollen; some die at one- or two-celled stages; and some form multicellular or multinuclear grains (see Fig. 1 and Table 2).

In barley, eight-celled pollen grains are formed from uninucleate microspores after six days of culture (Fig. 1). Some of them have nuclei in mitosis and are alive and healthy when taken from culture; others were dead or dying. The causes of embryo abortion are not well understood. The obvious possibilities are: inadequate diffusion of nutrients; presence of toxic products from the dying anther wall; chromosome errors arising from various causes; in hybrid material, action of deleterious recessives segregating after meiosis. The high death-rate at each stage makes it difficult to establish the exact pathway(s) of development of microspores that grow further into embryos or callus.

On the basis of work with *Datura* and *Nicotiana* species, where androgenesis is under good experimental control, SUNDERLAND (1974) distinguishes three routes to embryogenesis. In route A, the microspore passes through the normal polarized first pollen mitosis to form a vegetative cell with a large diffuse nucleus and a generative cell with a small compact nucleus. Then the vegetative cell, and afterwards its derivatives, divide repeatedly till an enlarged multicellular pollen grain is formed. These cells have nuclei resembling that of the original vegetative cell. The generative cell can also divide but only to a limited extent and the derivatives eventually abort. In route B, the microspore enters a non-polarized mitosis to give two cells of roughly equal size with diffuse nuclei. Then one or both continue to divide to give a multicellular grain. Route C begins with a normal polarized first pollen mitosis, as in route A. It is distinguished from route A by the participation of the generative cell in embryogenesis. The wall between the cells breaks down, the generative nucleus enters mitosis simultaneously with a vegetative nucleus, and they divide on a common spindle to give two equal diploid cells. It is supposed that these multiply to give a diploid pollen embryo. Variations of route C can lead to triploid or tetraploid embryos. At present route C has been established only for *Datura innoxia*.

Nuclear fusion is not confined to pollen grains on route C. In *Datura*, haploid nuclei on route B sometimes fuse to give diploid nuclei and it is at least possible that vegetative nuclei on route A can fuse, although this has not been demonstrated. Diploid and tetraploid nuclei can also arise without fusion, by endomitosis or endoreduplication. These events are common in callus cultures (see SUNDERLAND, 1973a) and are believed to account for some of the non-haploid plantlets from rice anther culture (NIIZEKI and OONO, 1971).

Route A as well as route B seem to occur in wheat (OUYANG *et al.*, 1973) and probably in *Lolium and* barley (CLAPHAM, 1971), and route A occurs in rice (IYER and RAINA, 1972). In *Triticale*, SUN *et al.* (1974a) have described a pathway that can be regarded as a form of route B. The cells or more often free nuclei arising from the first pollen mitosis are physiologically different even when the nuclei are of the same size. One nucleus, showing properties of the normal generative cell,

divides 1–4 times to form up to 16 free nuclei that stain intensely with Feulgen. The second nucleus, surrounded by cytoplasm that stains intensely with pyronine, after some delay divides repeatedly with wall formation to give the multicellular pollen grain. The free nuclei are pushed against the pollen wall and lost when the exine and intine split (Fig. 1). The same authors report that route A also occurs in *Triticale;* the development is of the same type except that nuclei after the first pollen mitosis are unequal in size.

In species such as barley and rye, where diploid and higher ploidy mitoses are seen at early stages, it is likely that a variation of route C is involved at least sometimes, but this has not yet been demonstrated. THOMAS and WENZEL (1975b) have found a three-celled grain in rye anther culture containing two triploid nuclei of vegetative type in mitosis and a generative-type nucleus in interphase (Fig. 1). This stage could be reached either by endomitosis and/or fusion of vegetative-type nuclei in route A, or, assuming two generative-type nuclei were originally formed, by a variation of route C (less probable). Triploid plantlets were in fact obtained in addition to the diploid and tetraploid ones.

The origin and future of the multinuclear grains often seen in cereal anther culture (Fig. 1) are not well understood. Some may arise in vivo from irregular cleavage at meiosis (SUNDERLAND, 1974) but others probably arise in vitro from normal microspores by free nuclear division. In barley, pollen grains with up to 30 free nuclei are formed but they degenerate after 15 days of culture (DELANNAY, personal communication).

Figure 1 shows in vivo pollen grains and extra divisions induced by B chromosomes in *Sorghum purpureo-sericeum* (DARLINGTON and THOMAS, 1941). After a normal but delayed first pollen mitosis, the vegetative cell could divide repeatedly till 3–5 generative cells were piled up against the wall opposite the germ-pore. Deviations from this pattern, ascribed by the authors to shifts of polarization, could give multicellular grains resembling those seen in cereal anther culture (e.g. Fig. 1N). Nuclear fusion was also reported (Fig. 1P). These authors speculate on the causation of what they call "encapsulated tumors".

2.1.3 Pollen Embryos, Pollen Calli, and Somatic Calli

Some of the unaborted multicellular pollen continue to grow and organize into structures that can reasonably be called "pollen embryos", particularly as the early development of the true cereal embryo is quite flexible (MAHESHWARI, 1950). Others, after breaking through the pollen exine and intine, form a less organized "pollen callus" (see Fig. 2).

It is highly desirable if pollen embryos rather than callus are formed, since it reduces the opportunity for chimaeras and also for the chromosome aberrations. The ideal situation for the breeder is the production of doubled haploid embryos, as *in Datura innoxia* (SUNDERLAND, 1974), and perhaps infrequently in rye (THOMAS and WENZEL, 1975b).

Anther somatic tissue, e.g. the filament, also forms callus in some species (such as wheat, OUYANG *et al.*, 1973; BAJAJ, 1976; rye, WENZEL and THOMAS, 1974). It is usually, but not always, obvious that this is happening.

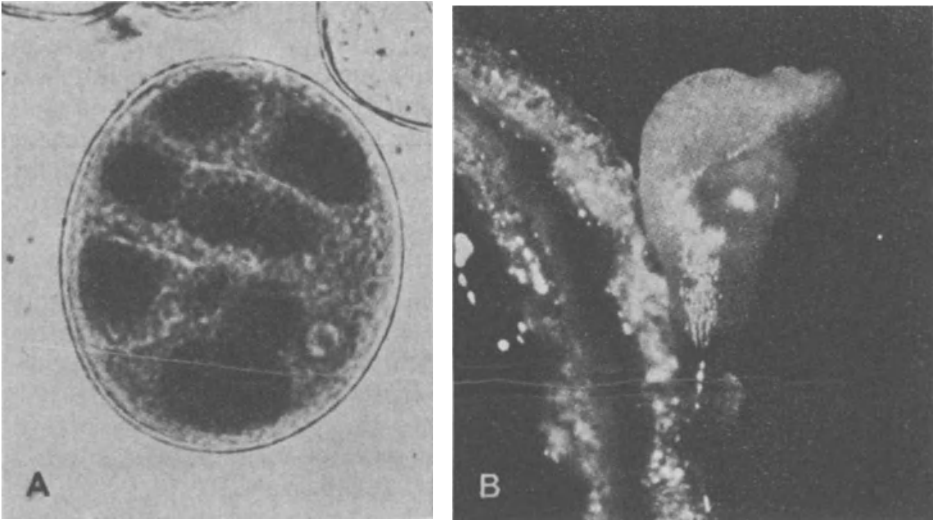


Fig. 2 (A) Multicellular pollen grain in rye (WENZEL and THOMAS, 1974). (B) Rye pollen embryo, with scutellum and coleoptile, after bursting through the anther wall. (THOMAS and WENZEL, 1975b)

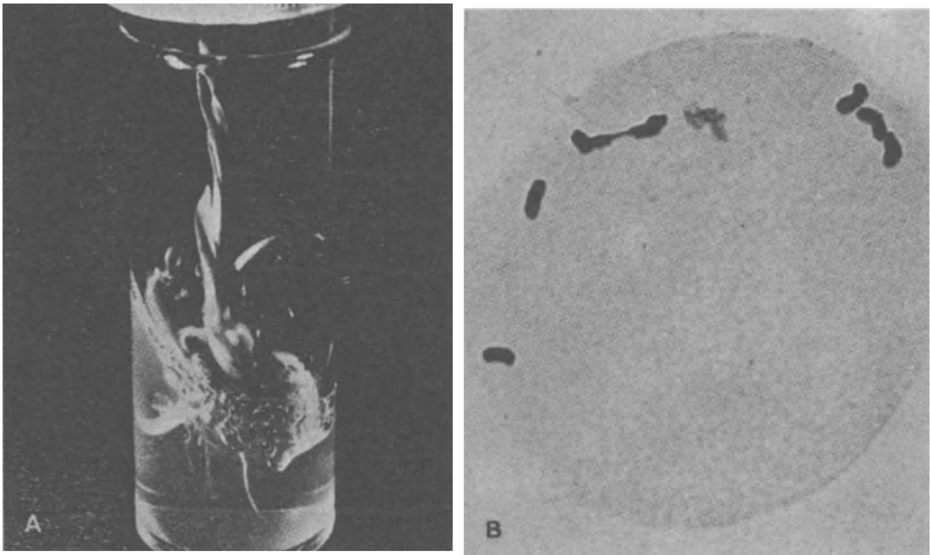


Fig. 3 (A) Barley plantlet from anther culture. (B) Meta-anaphase of meiosis in haploid barley from anther culture, showing five univalents and a bivalent. (CLAPHAM, 1973)

2.1.4 Testing for Homozygosity of Diploid Plantlets from Anther Culture

The origin of the diploid plants is important because of the varying genetic consequences. Diploid plants from the anther somatic tissue of a hybrid plant have the same hybrid genotype, and are of no interest. Diploid plants doubled

from normal haploid pollen are presumably homozygous and are certainly of value. There is, however, another possible category of diploid plants, those derived from unreduced pollen following total or partial suppression of meiosis (SUNDERLAND *et al.*, 1974). Methods of suppressing meiosis include failure of first or second anaphase, or reunion of two daughter nuclei at second telophase. Provided that pairing and crossing-over take place at the first meiotic division, the eventual diploid microspores are not only heterozygous but are of genotypes differing from each other and from the parent plant (DARLINGTON, 1965). To distinguish the desirable homozygous diploids from the undesirable heterozygous ones is easy for self-pollinated species. It is only necessary that the parent plants were heterozygous for genetic markers. Then the plants derived from anther culture are selfed and the progenies examined for segregation. Such experiments have been done with rice, barley and wheat.

In progenies of 16 diploid pollen plants from F_1 hybrid rice (2nd Division, 1974) 15 were strictly uniform and were presumably homozygous. The 16th segregated for plant height and panicle form, but the segregation was far simpler than in the progeny raised from seed from the F_1. Probably mutation(s) occurred in diploid cells in vitro.

WOO *et al.* (1973) obtained six diploid plants from cultivars Indica × Japonica rice hybrids. The phenotypes differed slightly among themselves and at first the plants were thought to be of pollen origin (WOO and TUNG, 1972). But five of them set seed, and the progenies were non-uniform, showing segregation for four characters. Therefore, it was interpreted that these five diploid plants were in fact heterozygous and derived from somatic tissue, and the original slight phenotypic differences were ascribed to cytoplasmic effects. (An alternative possibility is that the plants were derived from unreduced diploid pollen, see above.) The sixth diploid plant was sterile, but had properties making it plausible that it was derived from normal pollen (see also NIIZEKI and OONO, 1971).

In progenies from 18 diploid pollen plants obtained from hybrid barley and examined for segregation (JONES and PICKERING, 1973), all but one gave every indication of being completely homozygous. The one exceptional progeny segregated for a low temperature chlorina mutation but was otherwise highly uniform (see Fig. 4).

OUYANG *et al.* (1973) studied a fertile plant derived by anther culture from a hybrid wheat, and demonstrated exhaustively that the progeny was sufficiently uniform to be considered homozygous. The authors implied that chromosome doubling occurred during the callus phase in vitro rather than later in the field.

To conclude: diploid plants from cereal anther culture, unless clearly derived from the filament, are on present evidence usually doubled haploids and homozygous, but caution is needed. The issue is important, since nearly half of the rice plants (2nd Division, 1974) and more than half of the barley plants (CLAPHAM, 1973) are diploid.

2.1.5 The Albinos

Why do so many albinos, i.e. chlorophyll-deficient shoots, appear in cereal anther culture? Table 2 shows that they are frequent in *Aegilops*, *Hordeum*, *Oryza*, *Secale*,

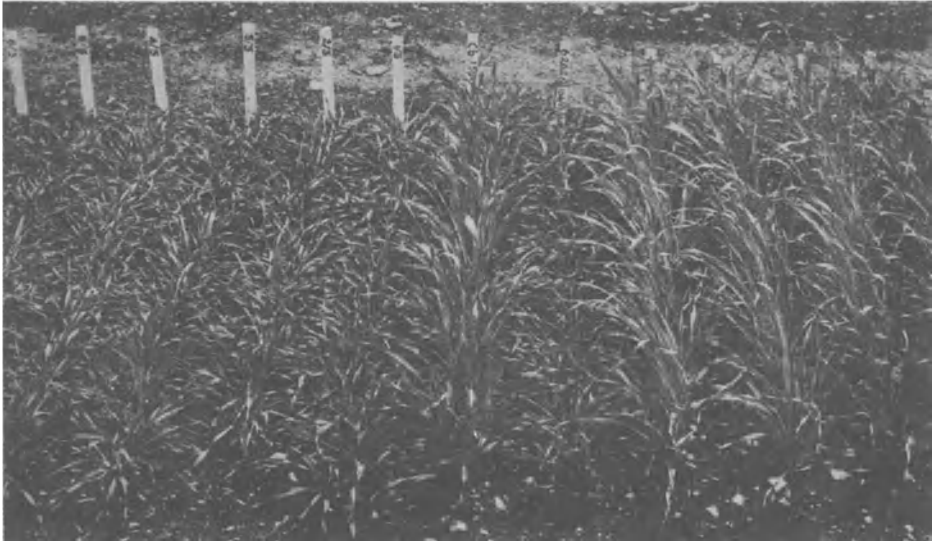


Fig. 4. Photograph showing the origin of diploid plantlets from normal haploid microspores of barley. The diploid plantles were derived from anthers taken from hybrid barley; their progenies, shown here, are each highly uniform but differ from each other with respect to segregating marker genes. (Courtesy of the Welsh Plant Breeding Station)

Triticale, and *Triticum*. The albinos of barley and rice can be white, yellow, light green and striped or chimerical in various ways. In barley (CLAPHAM, 1973) and rice (SUN *et al.*, 1974b) they have been studied electron microscopically. Some sections through an *albina* and a *xantha* barley plant unexpectedly showed virtually normal chloroplasts, with well-developed grana; these were nevertheless either inactive photosynthetically, or lay in patches of normal tissue. More in accordance with expectation, other sections showed only proplastids, with globuli and sometimes starch grains and concentric lamellar systems, but without grana. With the rice albinos, nothing more developed than proplastids with concentric lamellar systems, the authors commenting particularly on the absence of ribosomes from the plastids; the rest of the cell was normal. A first point is that the albinos do contain plastids, so that they have not originated from a cell completely lacking in plastids, as may be true of the generative cell.

Following are some of the possible suggestions for the origin of the albinos:

1. Pollen carrying chlorophyll mutations preferentially form plantlets in vitro. In barley, the spontaneous rate of chlorophyll mutation was found to be about six per 9000 spike progenies (NYBOM *et al.*, 1956). This indicates, on certain assumptions, an upper limit of about 3.3 chlorophyll mutations per 10000 gametes. Such a rate is just high enough to make it possible (though unlikely) that pollen carrying chlorophyll mutations form the albino plantlets in barley anther culture. However, such an hypothesis cannot account for the albinos from wheat anther culture, since wheat, being a hexaploid, displays spontaneous chlorophyll mutations at an enormously lower rate—too low to measure accurately. It is

therefore reasonable to exclude "mutation" in the ordinary sense of the word as the main cause of albinism in cereal anther culture.

2. The appearance of the proplastids in barley and rice albinos recalls the regression of the plastids during meiosis, described for *Lilium longiflorum* by DICKINSON and HESLOP-HARRISON (1970). The plastids regress between leptotene and pachytene, losing the greater part of the lamellar systems and most of the ribosomes. The mitochondria disorganize and at the same time the ribosomes are lost from the ground cytoplasm of the pollen mother cell. Plastid lamellar systems and ribosomes reappear at late tetrad and early microspore stages. It is possible that this re-organization is complete at a later stage of pollen development in cereals than in *Lilium*, at least for a fraction of the pollen, and is inhibited in vitro. SUNDERLAND (1974) has noticed that in barley cv Sabarlis grown at 20° C, 15–20% of the pollen grains just before anthesis (in vivo) are small, with little stainable cytoplasm, and were retarded in development. Similar pollen dimorphism occurs in *Petunia*, and in rye (WENZEL and THOMAS, 1974).

3. Another possibility is that the plastids disorganize in vitro. Ultrastructural studies on tobacco pollen show that during the initial inductive period of anther culture the plastids in the vegetative cell undergo a fundamental re-organization; it is suggested (SUNDERLAND, 1973b) that errors could occur at this stage that are not corrected. However such a process does not occur in *Datura* (DUNWELL and SUNDERLAND, 1974) and has not been studied in cereals.

The peculiarities of pollen should not be overstressed, since cultured Gramineaceous cells in general are liable to albinism. Thus GAMBORG *et al.* (1970) obtained exclusively albino plants from a somatic cell culture of *Bromus inermis;* and WENZEL and THOMAS (1974) obtained 50% albinos from the anther filament of rye. In other families too it is often difficult to obtain green somatic callus cultures (see EL HINNAWY, 1974). This would direct attention more to the conditions of culture. Unfortunately attempts to raise the frequency of green plants by alterations of the culture medium have not so far been very successful with cereal anther culture (CLAPHAM, 1973; DALE and HUMPHRIES, 1973).

2.1.6 Approaches to Cereal Anther Culture

Success with anther culture is strongly dependent on the genotype of the parent plant (GUHA-MUKHERJEE, 1973 on rice; BAJAJ, 1976 on wheat). It may be possible to exploit a genotype that responds favorably to anther culture by using it as one of the parents in a cross with the hope that the haploids are easily obtained from the hybrids as well.

There have been various proposals for adjusting the physiological status of the parents before anther culture. PICARD (1973), who emphasizes the importance of route B development in which the first pollen mitosis is non-polarized and results in two equal cells, recommends cold shocks to the wheat spike before anther culture. This is believed to promote the non-polarized mitosis and formation of pollen callus. An alternative approach is to spray plant growth substances on the plants before anther culture (PANDEY, 1973). Ethrel (2-chlorethylphosphonic acid), a source of ethylene and used as a male-sterilizer in several crop plants, can

induce additional mitoses in wheat (BENNETT and HUGHES, 1972), rice (WANG *et al.*, 1974) and *Petunia* pollen in vivo (BAJAJ, 1975).

As regards modifications of the culture medium, the inclusion of activated charcoal has recently been recommended for anther culture (NAKAMURA and ITAGAKI, 1973; ANAGNOSTAKIS, 1974; BAJAJ *et al.*, 1976). Eventually anther culture can be expected to give way increasingly to pollen culture (NITSCH, 1974a); the recent work of WENZEL *et al.* (1975), in which isolated rye microspores developed into multicellular structures, is the first success of this kind with cereals.

2.2 Other Means of Haploid Induction in Cereals

The following brief account is an attempt to put anther culture in perspective as a means of raising haploids in cereals. It is mainly concerned with work appearing since the reviews of KIMBER and RILEY (1963) and MAGOON and KHANNA (1963).

2.2.1 Use of the Cross *H. vulgare* × *H. bulbosum*

This valuable technique is reviewed by JENSEN (Chap. II.4 of this vol.). BARCLAY (1975) has recently obtained haploid wheat plants at high frequency after crossing *Triticum aestivum* cultivar Chinese Spring with *H. bulbosum.*

2.2.2 Use of Maize "Stock 6" and Its Derivatives in Conjunction with Genetic Markers

Work on maize haploids is well developed (CHASE, 1969). The spontaneous rate of haploidy in maize normally varies from 0.1 to 1 per thousand depending on the material examined (RANDOLPH, 1940); however, COE (1959) discovered a genetic strain, "stock 6", that on selfing gave the very high rate of 343 haploids per 10,616 plants (3.2%). An important feature of this strain is that it can be used as pollen parent for inducing haploids in other genetic material. For example SARKAR *et al.*, (1972) developed a derivative of "stock 6" suitable for certain conditions that induced a haploid frequency of 1.65% on four diverse female lines. The haploids were recognized by screening for an appropriate genetic marker. An effective method applicable when the female line is homozygous for factors determining colored aleurone and colored scutellum was to cross with the "stock 6" derivative (homozygous for the color inhibitor factor C^1) and then screen for kernels with colored scutellum and colorless aleurone. These are the haploids, in which the embryo has developed parthenogenetically and the endosperm after fertilization (COE and SARKAR, 1964). This method does not require the seed to be germinated in order to identify the haploids, so that thousands of kernels can easily be screened. Even if only one in 200 are picked out as haploids, the method is thoroughly practical. An effective means of doubling maize haploids has, however, yet to be found (CHASE, 1974).

2.2.3 Androgenesis and the "Indeterminate Gametophyte" Mutation in Maize

KERMICLE (1969) has discovered an interesting mutation *ig* "indeterminate gametophyte" in a highly inbred strain, Wisconsin-23. About 3% of the embryos in *ig*

embryo-sacs are paternal haploids. Such androgenetic haploids differ from those obtainable from anther culture in that the cytoplasm comes from the embryo-sac. Their special use is discussed in Section 3.4. The rate of spontaneous androgenesis in maize is otherwise very low, about one in 80000 (CHASE, 1969).

The general value of the *ig* mutation is unknown. The rate of androgenesis is strongly dependent on the genotype of the pollen parent, although the *ig* mutation is strictly sex-limited to the female parent and acts only in the embryo sac. Other effects of the *ig* mutation are: (a) half the kernels on *ig ig* plants and one quarter on *ig ig* have higher than normal endosperm ploidy level, (b) about 6% of the kernels with normal endosperm have more than one embryo.

2.2.4 Effect of *Aegilops* Cytoplasm on Cultivar Salmon of *Triticum*

KIHARA and his associates (see TSUNEWAKI *et al.*, 1974) have found that the cytoplasm of *Aegilops caudata* and five other *Aegilops* species induce at very high frequency haploids (11–56%) and twins (0.5–15%) in the progeny of a bread wheat variety, Salmon. The large majority of the twins were haploid-diploid. The special feature of Salmon wheat is that it is a *Triticale* derivative, overwhelmingly *Triticum*, but with a small part of chromosome 1B including the nucleolar organizer replaced by a piece of rye chromosome 1B, and two other minor changes. Single and twin haploids arise from the parthenogenetic development of the egg-cell; the diploid partner of a haploid-diploid twin arises by fertilization of a synergid. The plants with *Aegilops* cytoplasm and Salmon nucleus were produced by successive backcrossing of the initial hybrid to Salmon as pollen parent. They are male-sterile but female-fertile. The frequency of haploids in the progeny depends on the pollen parent used, as so often in haploid parthenogenesis, but is always unusually high. Unfortunately it is not easy to exploit the special features of this system.

2.2.5 Twins

A favorite old method of finding haploids is by searching among twin seedlings. In cereals this is not very effective. Even the case of *Aegilops*-Salmon wheat described above is not an exception, since the haploids are more frequent as singles than as twins. KAWAKAMI (1967), in a large survey of cereal twins, quotes frequencies of twins from his own results as 0.034% in *Triticum* (Einkorn, Emmer and common wheat), 0.032% in *Hordeum*, 0.227% in *Secale*, 0.059% in *Avena*, and 0.019% in *Oryza*. These were nearly always of the diploid-diploid or diploid-triploid type. The highest frequency of haploid-diploid twins occurred in *Triticum* (1 in 25) compared with 1 in 200 for *Avena* and none for *Secale*.

2.2.6 Colchicine-Induced Somatic Reduction

FRANZKE and ROSS (1952) reported that "Experimental 3", a partially inbred selection from an interspecific *Sorghum* cross, gave a number of true-breeding plants after treatment with colchicine. These proved to be doubled haploids (see KASHA, 1974b). It was also possible under special conditions to raise undoubled

haploids. For example, CHEN and ROSS (1963) treated 20 diploid derivatives of "Experimental 3" with colchicine under red light at 20° C and high humidity. Three out of the four survivors were haploid. Similar colchicine effects have been reported for the barley cultivar, Decatur (GILBERT, 1963). Since special colchicine-reactive cultivars are required, the phenomenon seems to be of limited application.

2.2.7 Fluorophenylalanine-Induced Reduction

Para-fluorophenylalanine is used to induce haploid sectors in diploid colonies of certain fungi, such as *Aspergillus niger* (LHOAS, 1961) and *Ustilago violacea* (DAY and JONES, 1971). Its effects have also been studied in higher plants (see Chap. II.1). NITSCHE (1973) treated hybrids of *Festuca pratensis* × *Lolium multiflorum* ($2n = 4x = 28$) with 3-fluorophenylalanine and examined root tips for chromosome counts. Cells most frequently contained 21 chromosomes, but sometimes as few as seven. Cells with anomalous counts were still present four months after treatment. Fluorophenylalanine is worth further study with cereals.

3. Uses of Haploids in Cereal Breeding

3.1 Production of Homozygous Varieties in Self-Pollinated Cereals

The obvious application of haploid techniques to cereal breeding is as a quick route from heterozygotes to homozygotes (EAST, 1930). In self-pollinated crops, this is usually done by five to seven generations of self-fertilization. The alternative is to raise haploids from the heterozygous material and then double the chromosome number by colchicine or otherwise. The resulting diploid plants are strictly homozygous except in as much as colchicine may promote mutation. The conventional method takes three years, even if two generations can be grown each year, whereas the homozygotes are available in one year if haploids are used.

Whenever breeding program begins with a cross to give hybrid material from which pure or nearly pure lines must be derived, as is usually the case, the haploid method appears to offer advantages. Yet plant breeders are not always convinced. It is best to compare briefly the merits and shortcomings of established methods with those of haploid techniques. The established approach of the pedigree method is discussed here.

In the pedigree method, selected parents are crossed, the F_1 seed grown, and then succeeding generations raised by self-pollination till a high degree of homozygosity is attained in about the F_7. The plants are spaced to permit strong selection from the F_2 onwards, at first on a single plant basis to eliminate genotypes with undesirable major genes, later on a family basis to improve complex characters such as yield. The important feature of the pedigree method is the way in which selection, assessment and to some extent multiplication of the new potential variety is carried out while at the same time the material becomes more homozygous. An unpromising cross can be abandoned completely at the F_2 stage.

Drawbacks of the method are that it is time- and land-consuming, and the breeder tends to be confused by heterozygous advantage at the early stages of selection. The drawbacks can be reduced by using the compromise method outlined by MAC KEY (1962), in which selection is postponed till the F_4, followed by reselection at a later stage.

The procedure with haploid techniques might be as follows. The initial cross is made, the F_1 plants grown, haploids are derived from the F_1 and doubled to give homozygotes. At this stage some selection for simple characters may be possible, but normally the doubled haploids must be allowed to set seed and the progenies examined; otherwise the effects of colchicine or the tissue culture origin of the plants will interfere with assessment, which in any case is very difficult on a single plant basis. In fact, if seed on the doubled plants is set in the greenhouse, it may be best to grow another generation in the field before comparing with control varieties, raised from seed set in the field (WALSH *et al.*, 1973). If this is done, the time-saving potential of the haploid method can be reduced but not eliminated.

What do the different approaches offer? The haploid technique probably saves two or more years as compared with the pedigree method. In terms of labor, the advantage is less obvious, although quantitative comparisons of the methods are hard to make. NEI (1963) in his interesting analysis begins by supposing that the initial cross differs with respect to n genes, presumed unlinked. He then points out, in effect, that a minimum of 2^n plants must theoretically be grown for all homozygous genotypes to be represented using haploid techniques, whereas 4^n is the corresponding figure using conventional breeding methods. This does not really do justice to the pedigree method, the essence of which is that the unpromising plants or segregating families are rejected in the early generations of selfing, thus removing the need for growing all genotypes.

If, for example, the parents in the initial cross differed by as few as ten genes, presumed unlinked, a minimum of $2^{10} = 1024$ doubled haploids would have to be grown for the chance occurrence of all segregants, including the optimal one. KASHA (1974b) estimates that with the *Hordeum vulgare* × *H. bulbosum* technique, 100 doubled haploids of barley can be raised per worker per week, so that one person would have to work for 2½ months. Far more plants need to be grown using the pedigree method, but handling them is for the most part easy and rapid. In practice, a breeder wishes each year to test many crosses, in each of which a hundred or more genes are segregating. There is then no chance of securing optimal combinations of genes; it is rather a matter of rejecting most of the material with a minimum of effort. In such circumstances haploid techniques are too laborious. They have the further disadvantage, as compared with the pedigree method, of restricting the number of opportunities for tight linkages to be broken by crossing-over. For a fuller analysis arriving at broadly similar conclusions see WALSH (1974).

Haploid techniques have perhaps most potential when selecting for characters that must be assessed by a complicated test, such as a chemical analysis for quality, and when the character is under the control of a number of genes with dominant effect (MELCHERS, 1972). These circumstances are, however, rather specialized. Haploid techniques might also conceivably be used in the breeding of homozygous parents for hybrid varieties of wheat and barley (MELCHERS, 1972).

The newly-developed doubled haploid stocks would initially be highly uniform, a possible attraction; but in the course of seed multiplication, owing to mutation they are likely to become as variable as ordinary inbred lines.

3.2 Production of Hybrid Varieties in Cross-Pollinated Cereals

The first step in the breeding of cereals such as maize and sorghum is usually the derivation from heterozygous material of pure lines for use as parents of the intended single-cross or double-cross hybrids. The haploid method can be used to produce these pure lines with considerable saving of time. In fact, with maize there is much practical experience of using the haploid method, described by CHASE (1969). It has been employed successfully (CHASE, 1974) in a commercial plant breeding venture, even without the benefit of a high-frequency haploid-inducing line such as "stock 6". It can be expected to become more popular with maize breeders now that such lines are available.

Criticisms sometimes made of haploid techniques are:

1. With haploid techniques, there is only one opportunity for intrachromosomal gene recombination to occur, whereas with conventional inbreeding it can occur at each generation of selfing. CHASE (1969) writes however: "It is quite clear from theoretical considerations that if intrachromosomal gene recombination is required, it is more efficient to provide opportunity for recombination to occur before inbreeding is initiated than during the inbreeding process." This drawback of rapid inbreeding is recognized by maize breeders, who frequently adopt the procedure known as "recurrent selection". This is intended to allow maximum opportunity for recombination and to concentrate favorable genes. Cycles of crossing among selected parents and reselecting among the progeny are interposed between successive generations of self-pollination (see ALLARD, 1964). This makes the production of inbred lines into a lengthy thoughtful process, to which the rapid haploid technique may be preferred.

2. Cross-pollinators tend to resist rapid approaches to homozygosity, owing to the segregation of deleterious recessives and to the other causes of inbreeding depression. Probably for this reason, it initially proved very difficult to raise haploids of rye unless inbred lines were used as starting material (NORDENSKIÖLD, 1939). Similarly with maize, haploids are easier to induce in inbred than in heterozygous material (CHASE, 1969). It is therefore sometimes claimed that the haploid method is too extreme for use with cross-pollinators. Alternatively one might suppose that a technique such as anther culture is very effective at picking out the few viable combinations of genes that can be fixed in homozygous diploid form. Indeed recently, doubled haploids have been obtained from hybrid rye by anther culture (THOMAS and WENZEL, 1975b).

3. SPRAGUE (1967) depreciated haploid techniques by claiming that the problem in maize breeding is the recognition and testing, not the production, of inbred lines suitable as parents of hybrid varieties. However CHASE (1969) thinks that breeders too often laboriously accumulate unmanageable numbers of unremarkable inbreds when they would be better advised to try for excellent ones rapidly via haploid techniques.

3.3 Use of Haploids in Mutation Breeding

The use of haploid cells and protoplasts for mutation experiments is discussed in Chapter V of this volume. Alternative approaches to haploid mutation breeding are: (1) to induce mutants by treating florets with acute doses of X- or γ-radiation, or with chemical mutagens before anther culture, (2) to incorporate a chemical mutagen into the culture medium, (3) to treat haploid plantlets with an acute dose of a mutagen (DEVREUX and DE NETTANCOURT, 1974) or (4) to grow haploid plants in a gamma field and expose to chronic gamma radiation (TANAKA, 1970). Approach (1) has the advantage of avoiding the formation of chimeras that will arise if whole plants are treated with a mutagen.

The obvious advantage of haploids is that they display mutations with recessive effects in single dose. This is a very important point if mutagenesis and selection is being applied to cell cultures. Otherwise the advantages of haploid techniques in cereal mutation breeding seem to lie particularly with the ease of selecting micromutants from the genetically homogeneous M_2 progenies obtained from doubled, mutated haploids, as compared with the genetically heterogeneous M_2 progenies if the mutagen is applied to diploids.

To illustrate, TANAKA (1970) has experimented with haploid rice plants multiplied vegetatively from a single genotype, and irradiated in a gamma field at various doses for a hundred days. Tillers propagated from buds that had been irradiated at an early stage of development frequently gave diploid panicles with fertile seed: 152 panicles with at least five fertile seed were obtained from 9315 plants derived from 440 irradiated plants. More than half of the radiation-induced diploid panicles gave pure lines, about 40% being mutant with small but significant changes of character. The conclusion is that irradiating haploid plants in a gamma field is an effective way of producing pure lines with micromutations.

The reason why nearly half of the radiation-induced diploid panicles gave genetically mixed lines, often segregating for gross mutations, is presumably because mutations were sometimes induced after diploidization. This clearly reduces the efficiency of the method, which remains, nevertheless, still considerable.

3.4 Miscellaneous Uses

CHASE (1952) suggested the use of androgenesis in maize as a technique for the one-step transfer of cytoplasm from one line to another. The idea is that an androgenetic haploid, originating from a male gamete (virtually devoid of cytoplasm) and developing without fertilization in the embryo sac, should have the pollen parent's nuclear genotype and the seed-bearer's cytoplasm (see CHASE, 1969). If the seed-bearer has cytoplasmically determined male sterility, the (doubled) androgenetic haploid should have the same. This has been confirmed experimentally by GOODSELL (1961) and KERMICLE (1973). On occasions, however, androgenetic plants are unexpectedly male-fertile instead of male-sterile, the reason for which is obscure (CHASE, 1969). Of course, androgenetic plants from anther culture are of no use here.

Of the many other uses of haploids in cereal botany, following are some of the examples. (1) A cytological study of haploids led to the discovery of the control of

disomic inheritance in wheat by chromosome 5B (see KIMBER and RILEY, 1963). (2) Haploid maize has been used in the study of the synaptonemal complex (GILLIES, 1974). (3) Further applications to cereal breeding are conceivable (e.g. 4x derivatives from 8x *Triticale*). (4) Doubled haploids from self-incompatible plants such as rye should be homozygous at the incompatibility loci—a condition hard to obtain by other means.

4. Conclusions

Progress is being made with cereal anther and pollen culture and a degree of success is reported for a number of species; however, the techniques need considerable further development. The yield of plantlets is usually low and unreliable; the pollen rarely organizes directly into an embryo, but passes through a callus phase, with the result that chromosome aberrations may arise; albinos rather than green plants too frequently differentiate from the callus. It is likely that in the near future some of these problems will be overcome. If doubled haploid embryos and plantlets can be obtained in reasonable yield direct from culture, as with *Datura innoxia* (SUNDERLAND *et al.*, 1974), the method would be particularly attractive as avoiding the need for later colchicine treatment.

Another in vitro method, very effective, is available for barley and wheat, and depends on chromosome elimination in the embryo resulting from a cross with *H. bulbosum*. In maize, parthenogenesis and androgenesis in vivo can be exploited as routes to haploidy.

Cereal haploids will probably make their greatest impact on plant breeding indirectly via cell and protoplast culture and advances in biochemical genetics. In the nearer future, haploids can be used effectively in the production of parents for hybrid varieties, particularly of cross-pollinated cereals. They also show promise in connection with mutation breeding and research.

Acknowledgments. I am grateful to the Editors, to Dr. T. Eriksson, and to my colleagues at the Dept. of Genetics and Plant Breeding for help with the manuscript, and to Dr. K. Delannay, Dr. N. Sunderland and Dr. E. Thomas for sending copies of their unpublished papers.

References see page 331.

4. Monoploid Production by Chromosome Elimination

C. J. JENSEN

1. Introduction

The production of monoploids in relatively large frequencies is of great value to breeders and geneticists. Where monoploids can be induced in crop plants they may be used to simplify patterns of inheritance and accelerate breeding programs as the quickest possible way to homozygosity. Monoploids constitute moreover an ideal material on which to base advanced genetics in higher plants (analogous to fungal or microbial genetics) and through cell culture faciliatate an understanding of the biochemistry, developmental processes and evolutionary aspects of higher plants.

The purpose of this account is to give the status on monoploid production by the chromosome elimination method in barley and the impact of in vitro culture on this achievement.

Agricultural barley (*Hordeum vulgare* L., sensu lata) is a diploid species ($2n=2x=14$). It is one of the oldest cereals known to man and ranks fourth in the world's cereal production (only surpassed by wheat, rice and maize). Barley is self-pollinating, annual and found as either two- or six-rowed, spring or winter types. Traditionally the barley grain has been used in human diet, animal feed, and in producing alcoholic beverages. Its conspicuous chromosomes, morphological characters, and ease of handling have made barley a valuable object for mutation studies. Genetically it is among the best-known higher plants (for a general review see FRØIER *et al.*, 1959; BELL and LUPTON, 1962; NILAN, 1964).

Monoploids are here defined as sporophytes with the basic chromosome number (i.e. the gametic chromosome number (DE FOSSARD, 1974). They have a single genome or chromosome set ($2n=x=7$). Monoploids are generally sterile but by doubling the chromosomes, homozygous diploid fertile plants are produced. They have been reported to occur spontaneously in barley (see JENSEN, 1974a, for references).

Monoploids have been found in a number of species (for a review see KIMBER and RILEY, 1963; CHASE, 1969, 1974; KASHA, 1974a,b). Although monoploid sporophytes are smaller in size than the corresponding diploid plants they need not be feeble plants. Their vegetative growth may be just as vigorous as the corresponding diploids. The may also have a long life-span as exemplified by the monoploid sporophyte of *Thuja plicata* ($2n=x=11$) (*T. gigantia* var. gracilis, Pohlheim, which has been a sporophyte for over 100 years). Like almost all monoploids it is completely sterile. A monoploid of horticultural interest is the *Pelargonium* cultivar "Kleine Liebling" ($2n=x=9$) which has been reproduced vegetatively over a number of years. Maize was the first crop where monoploids have been produced systematically and employed as doubled monoploids in breeding programs (CHASE, 1969, 1974).

Monoploids are an ideal tool in cell culture where they form a simple system (only one allele) in biochemistry and cell genetic analysis (ZENK, 1974). Their usefulness in cytogenetics has already been exemplified by SADASIVAIAH and KASHA (1971), and GILLIES (1974). Perhaps their use in in vitro studies (e.g. auxotrophic mutants) will become more important than their application in breeding programs and conventional genetics of higher plants. The use of monoploids in various disciplines will depend, of course, on the ease and reliability of their production. The present account deals with current practices of producing monoploids in barley.

2. Scope

Monoploids as sporophytes in barley ($2n = x = 7$) may be induced by altering the normal development of microspores (potential male gametophytes) or by utilizing the female gametophyte. They can best be produced by interspecific hybridization followed by chromosome (genome) elimination. The hybridization method consists of crossing cultivated barley, *Hordeum vulgare* L. ($2n = 2x = 14$), with the wild, diploid cross-pollinating and perennial *H. bulbosum* L. ($2n = 2x = 14$).

For practical purposes, monoploids have to be produced in relatively high frequencies from all possible genotypes of barley. The interspecific hybridization of barley with *H. bulbosum* allows for a production of relatively large numbers of monoploids. This technique, the Bulbosum method, consists of the following steps: the female gamete of barley is fertilized by the *H. bulbosum* gamete. Zygote and embryo induction is high, but during this process the chromosomes of *H. bulbosum* are eliminated, leaving the barley genome in the embryos. The latter are cultured in vitro as immature embryos. Plantlets from these monoploid embryos can be made to give fertile flowers bearing homozygous offspring following an efficient chromosome doubling technique.

Emphasis is placed here on an outline of current techniques in the production of monoploids, and on indicating that the Bulbosum method can be made attractive to serve breeders and geneticists by improving its stability and efficiency. Recent results on anther culture of barley are also given and discussed in relation to pollen or microspore culture methods.

The frequencies of spontaneous monoploids are far too low for utilization in breeding or genetic studies. If monoploids should become a tool in the various disciplines of plant sciences, applied as well as in basic research, methods must be available to allow a high frequency production from all possible genotypes without changing the genetic character of the original material.

3. Usage and Need for Monoploids

The advantages of monoploids as tools in plant breeding or genetics become more apparent when their direct application is visualized. Some of the advantages have already been listed (JENSEN, 1974a) as: (1) they provide the quickest possible

way towards complete homozygosis, (2) they may serve to recover recessives, (3) by sampling gametes as monoploids linkage data can be obtained directly, (4) doubled monoploids give an immediate product of stable recombinants from species crosses, (5) monoploids can be used to determine homology within a genome and between genomes, (6) they are ideal objects on which to study mutation frequencies and spectra, (7) in cell and protoplast culture they provide an ideal system for fundamental cell biological problems (i.e. biosynthesis),

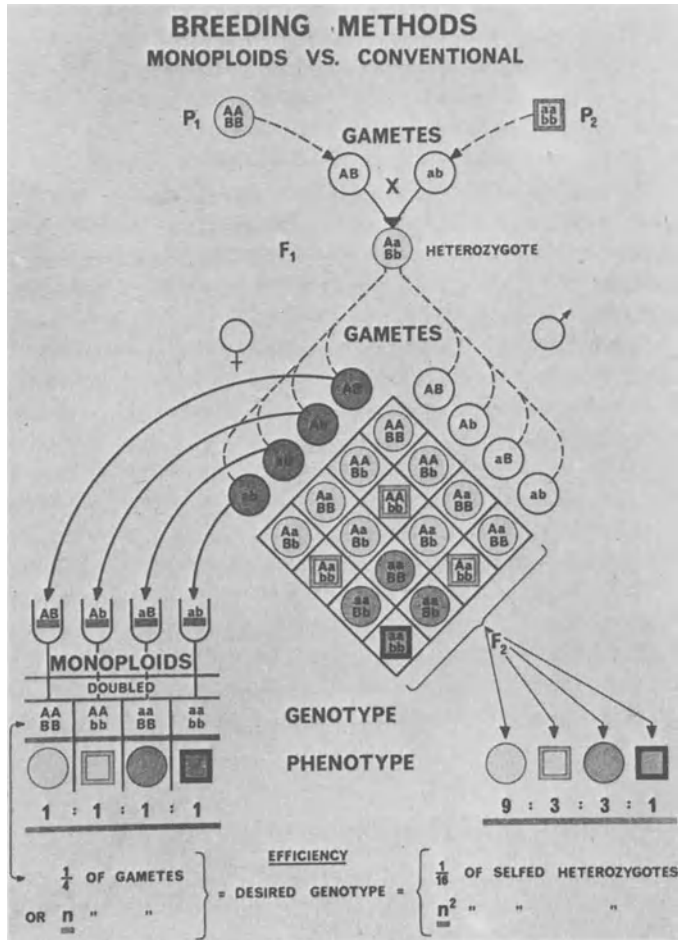


Fig. 1. Advantages of monoploids and doubled monoploids in breeding methods and in genetic analyses. By turning female gametes (from F_1 plants) directly into sporophytes and doubling their chromosome number, the segregation product is simplified. This relationship will simplify analyses when many different combinations of alleles are tested. By obtaining the homozygous condition from gametes directly a given breeding program aiming at homozygozity can be shortened by 5 to 6 plant generations. It can be visualized that there are decided advantages for the breeder and geneticist in applying the monoploid method to resolve possible recombination products by sampling and selecting on chromosome doubled gametes

(8) monoploid cells as protoplast provide a unique material for gene transfer; host–pathogen reactions; and cytoplasmic and/or chromosomal incompatibility.

Some of these advantages are of direct practical value (CHASE, 1969, 1974; MELCHERS, 1972; KASHA and REINBERGS, 1975). Figure 1 depicts this by comparing conventional breeding methods with the monoploid method, though somewhat simplified (see also WALSH, 1974; Chap. II.3).

For breeding purposes one of the main advantages of using monoploids is that completely homozygous lines are produced directly from gametes of F_1 hybrids or from later (advanced) selections. This allows for a direct fixation of quantitative characters (as used in studies on yield in high lysine mutants and on pleiotropic effects of mildew-resistant mutants and also on short straw mutants at Risø).

The utilization of monoploidy in genetics and breeding requires that a random sample of gametes is obtained and that the gametes are converted into sporophytes at relatively high frequencies.

In terms of practical plant breeding the most important aspect of using monoploids and doubled monoploids in barley is a saving of time in several plant generations and the ease of recognizing the desired product among lines for selection. These points have been described and discussed in more detail by PARK *et al.* (1974) and KASHA and REINBERGS (1975). REINBERGS *et al.* (1975) have provided evidence from a comparative study on field-grown barley lines that about 20 lines are sufficient to characterize a given cross. The material consisted of 52 doubled monoploid lines from two crosses which were compared with pedigree lines and lines obtained at random (single seed descent). The comparison which was made on two locations for two years clearly showed that the arguments brought forward for the monoploid method in maize (see CHASE, 1969, 1974) can also be brought forward for a self-pollinating species like barley. These workers found a decided advantage of the monoploid method in saving time for the breeder and providing a reliable material on which to base selections.

Other areas in which monoploids could be advantageously used are: seed of doubled monoploids would make a good material to start mutation studies in barley. Monoploid cell and tissue cultures, especially the protoplasts, will provide the geneticist, biochemist and cell biologist with a material that might prove a powerful tool in plant modification and somatic hybridization (see Chap. IV.).

4. Production of Monoploids in Barley

For the artificial induction of monoploids in barley there are now two routes which can be illustrated as follows:
(1) Based on potential male gametes (microspores, see also Fig. 10), and (2) on the female gametes (megaspores). Potentially the first route, via anther or microspore culture, has the advantage because there are far more potential monoploids per spike in the form of male gametophytes than there are female gametophytes. Both methods are based, however, on embryogenesis and the development of plants from monoploid embryos followed by chromosome doubling to obtain homozygous diploids.

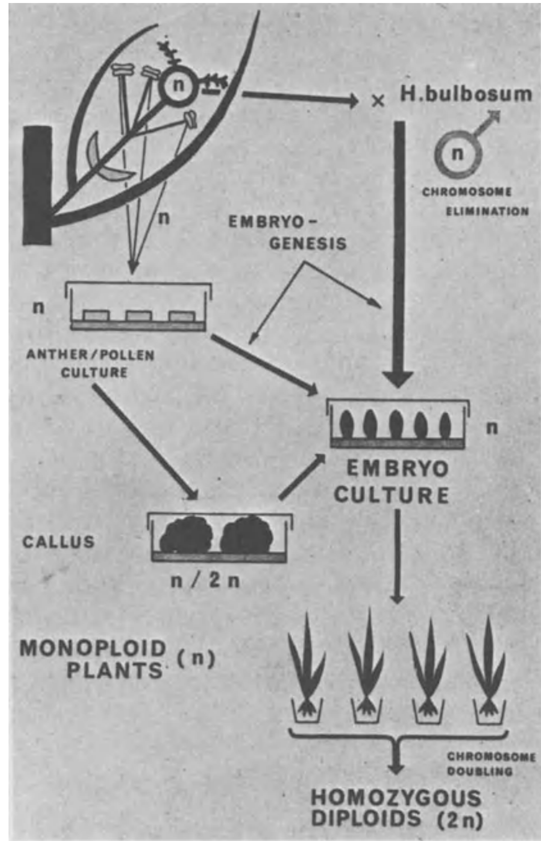


Fig. 2. Diagram relating two main routes of monoploid (n) and doubled monoploid production in barley. One route is sketched via anther or pollen culture and the other via use of the female gamete, which after fertilization by another *Hordeum* species and zygote formation, develops into a monoploid embryo by somatic chromosome elimination of *H. bulbosum* chromosomes

5. Anther and Microspore Culture

Anther culture has been described in barley by CLAPHAM (1971, 1973), GRESSHOFF and DOY (1972a), BOUHARMONT (1974), ZENKTELER and MISIURA (1974), MALEPSZY and GRUNEWALDT (1974), GRUNEWALDT and MALEPSZY (1975), PEARSON and NILAN (1975), DALE (1975, personal communication). It is evident from these reports that anther culture in barley has, at present, considerable limitations. There are still major obstacles to be solved before the anther or microspore cultures of barley can be as effectively handled for haploid production as those of the Solanaceae (see references in NITSCH, 1974b, 1975; SUNDERLAND, 1974; BAJAJ, 1976). These limitations derive from three main areas: the physiological condition, the growing of donor plants, and plant induction via callus. Nonsynchronous development of microspores at the culture stage in Gramineae (WENZEL *et al.*, 1975), and differential behavior of genotypes (GRESSHOFF and DOY, 1972b) lead to low frequency and variability in embryogenesis and organogenesis from callus and the formation of chlorophyll-deficient and karyotypically abnormal plants. If these limitations can be overcome this method would be potentially more attractive than the interspecific hybridization method.

6. Interspecific Hybridization—The Bulbosum Method

The alternative method of obtaining monoploids in barley is based on interspecific hybridization. The first report on utilizing hybridization and somatic chromosome elimination (see KASHA, 1974a) came from crosses between tetraploid *H. vulgare* ($2n=4x=28$) and tetraploid *H. bulbosum* ($2n=4x=28$). Soon after their first report on haploid barley induction (KAO and KASHA, 1969), KASHA and KAO (1970) demonstrated that the method of crossing diploid barley with diploid *H. bulbosum* could be used to produce high frequencies of barley monoploids. Although other reports had already pointed out the cytological consequences of this cross combination (SYMKO, 1969; LANGE, 1971), it was the application of embryo culture following growth of donor plants in controlled artificial environments, that made these relatively high frequencies of monoploid production possible. Following this report other workers became interested in the production of monoploids (JENSEN, 1973, 1974a; FEDAK, 1973; ISLAM and SPARROW, 1974; FINCH, 1974). This method is currently used by one private plant breeding firm in Canada and by more than a dozen major research institutions throughout the world. The technique is recommended as a tool in breeding and genetics and allows for: (1) Induction and production of large numbers of normal monoploids, (2) monoploids and doubled monoploids occur randomly from all possible genotypes (3) genetically unaltered and stable doubled monoploids (homozygotes) are made in the shortest possible time.

7. Principles of the Bulbosum Method

In essence the Bulbosum technique is based on making an interspecific cross with *H. vulgare* as the female and *H. bulbosum* as the male. The method utilizes the female gametes of barley and is schematically representd in Figure 3.

Fertilization of *H. vulgare* by *H. bulbosum* pollen proceeds readily. Zygote induction is high and the chromosomes of *H. bulbosum* are rapidly eliminated from the cells of the developing embryo. The endosperm develops for two to five days and then aborts. In the developing monoploid embryo cells the division and increment is slower than in diploid cells (JENSEN, unpublished). This comparatively slow growth of the monoploid condition, together with the disintegration of the endosperm, leads to the formation of small embryos which have to be dissected out of the fruits and provided with nutrients in vitro in order to complete their development. Following in vitro embryo culture the developing plantlets are raised under normal greenhouse conditions and chromosome doubling is induced on established plants.

The method has the advantage that very high frequencies of female gametes are induced to form embryos from which all *H. bulbosum* chromosomes are usually lost. Hybrids, although easily recognized, are normally rare. The mechanism by which chromosome elimination proceeds has not been established. However, it is genetically controlled by chromosomes two and three of *H. vulgare* (KASHA, 1974a; HO and KASHA, 1975).

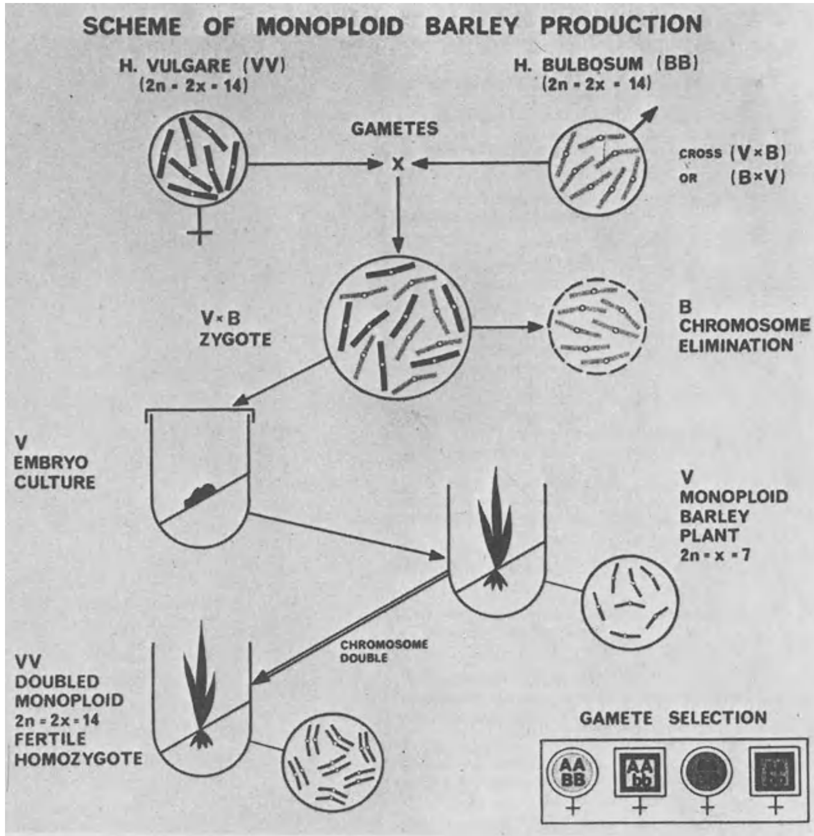


Fig. 3. Scheme of monoploid and doubled monoploid barley production via the interspecific hybridization method followed by somatic chromosome elimination of one of the parents chromosome sets, here *H. bulbosum*

8. The Technique of the Bulbosum Method

8.1 Growing Barley for Crossing

The *H. vulgare* to be used as female parents should be reared under the most favorable conditions for the respective genotypes. It is also important to maintain a continuity of flowering plants over a period. Figure 4a lists the main working steps in producing monoploids and doubled monoploids.

At Risø the seeds are sown in pots filled with a peat soil (commercial "K-soil") to which fertilizers have been added. Flats containing 24 pots are placed on glass-wool mats on low glasshouse benches. When the seedlings are established (about two weeks after sowing) water is supplied twice daily to the flats by an automatic drip and additional fertilizer is provided every two weeks. An 18 h-a-day light source consists of HQIL-Osram-400 W lamps. The light intensity at plant pot level

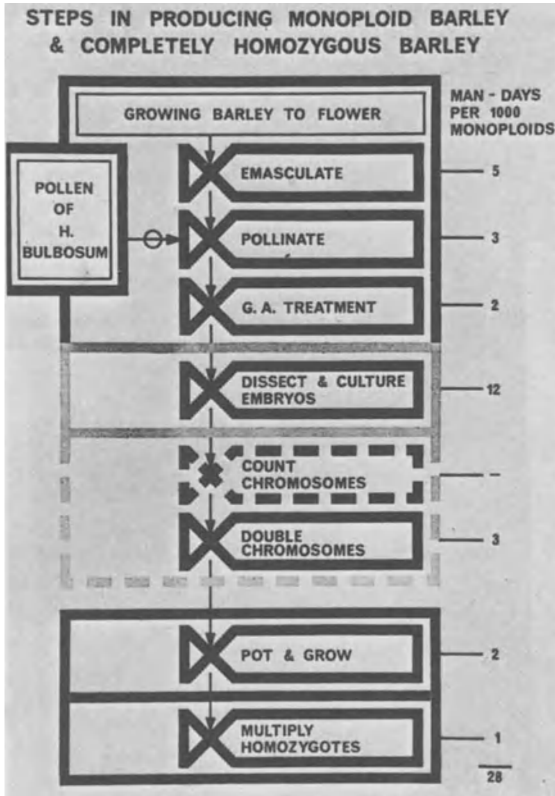


Fig. 4a. Steps in producing monoploids and doubled monoploids in barley. The various steps are listed in sequence of procedure (*G.A.* hormone treatment). Approximate figures in man-days per 1000 monoploids are given for the various steps indicating various grades of ease of execution

is 46×10^3 lux and at the level of the spikes about 84×10^3 lux, with three lamps per m^2 of growing area. STOSKOPF *et al.* (1970) give a description of growth-room facilities which permit control of environmental conditions throughout the year and provide relatively large growing areas. The day (18 h) temperature is about + 18° C and the night (6 h) temperature + 13° C. (Growing conditions at Risø are shown in Figure 6a, b.)

There is evidence that spring type barley adapted to one growing area (e.g. six-rowed Canadian cultivars) behave differently and need quite different temperature conditions than for example Northern European two-rowed cultivars. Examples of environmental influences on the genotype are given by GUSTAFSSON and DORMLING (1972), and DORMLING *et al.* (1975).

8.2 Hordeum bulbosum L.

Diploid *H. bulbosum* is a wild, perennial, cross-fertilizing species found in some hilly parts of Morocco, Tunisia, Cyrenaica, Italy, and Spain (LEIN, 1948; KATZNELSON and ZOHARY, 1967) and vernalization is needed to induce flowering (KOLLER and HIGHKIN, 1960). It is self-sterile (LEIN, 1948; LUNDQVIST, 1962), but it is easy to propagate plants vegetatively from bulbils produced at the bases of stems. At

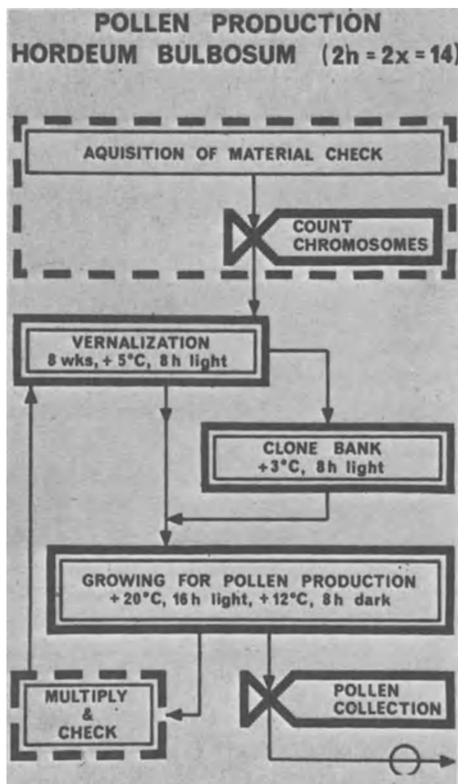


Fig. 4b. Scheme of handling *H. bulbosum* material

Risø we find it practical to plant *H. bulbosum* material out in the field in autumn. After a good year's growth in the field bulbs are taken in, potted and placed in a cool room. This material then gives good flowering shoots when moved to the crossing area. Figure 4b presents the scheme of handling *H. bulbosum* material. As expected with a wild, allogamous species there is considerable variation within families or ecotypes.

8.3 Emasculation of Barley

For ease of handling, the potted plants are removed to a crossing area where the florets can be emasculated in one of the conventional ways (Pope, 1944; Hamilton, 1953; Wells and Caffey, 1956).

1. Sideway (slit) removal of the anther with forceps, the forceps pierce the palea and lemma lengthways forming a slit through which the three anthers are easily extracted without damaging the stigma (Fig. 5b).

2. Cutting across the top third of the flower above the anthers ("egg-topping") with a pair of scissors, care being taken not to damage the stigma. If this is done in the afternoon the next morning the anthers will have begun to push through the cut opening and can easily be removed.

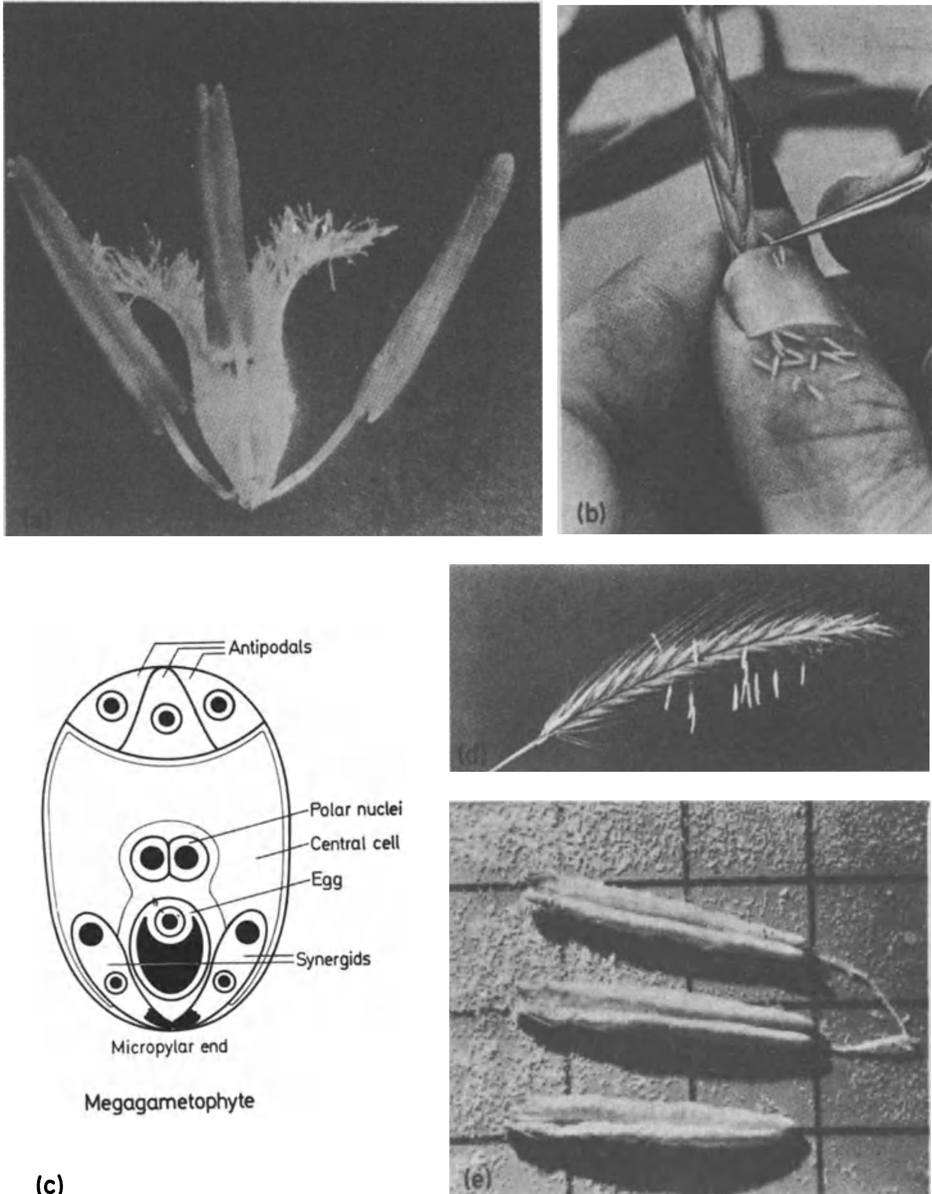


Fig. 5a–e. Stages of *Hordeum* floral structures for monoploid production: (a) Three anthers and ovary with stigma of a barley flower almost at the selfpollinating stage. (b) Hand emasculation of barley spike (*inset*: emasculated floret). (c) Highly schematised ovule of barley. (After JENSEN, 1973). (d) Flowering spike of *H. bulbosum* shedding pollen

The following procedures increase the level of seed set:

a) Emasculation is best done on fully mature flowers preferably about one day before natural pollen release. By leaving emasculation to a late stage increases the risk of accidental selfpollination but selfed fruits can easily be distinguished from

those of *H. bulbosum*-crossed fruits at harvest, as the latter are devoid of a proper endosperm (Fig. 6f).

b) On two-rowed cultivars four to six of the basal florets are left to self-pollinate, all other florets are emasculated except poorly developed ones at the tip of the spike (which is removed) in all about 25 florets per spike. Six-rowed cultivars are treated intact because removal of the outer florets damages the normal functioning of the spike.

c) After emasculation the spike is enclosed in a parchment bag which is in turn covered by a cellophane bag. Closing the base of the bag with a clip ensures the maintenance of a high level of humidity which is of particular importance for good fruit set (Fig. 6c).

8.4 Pollen Production and Collection in H. bulbosum

Unfortunately at present there is no method to store *H. bulbosum* pollen satisfactorily. Thus, an ample supply of freshly collected pollen has to be available throughout the crossing period. To achieve a continuous supply of pollen, vernalized plants of *H. bulbosum* are moved at regular intervals from the cool room to the glasshouse or growthroom.

Normally one *H. bulbosum* plant is needed to pollinate two to five barley plants. However, this depends on the productivity of the *H. bulbosum* material, on its mode of flowering and on the timing of the flowering peaks. The best pollen-producing plants are obtained from large, well formed bulbs. At Risø plants producing these bulbs are obtained from field-grown *H. bulbosum* material.

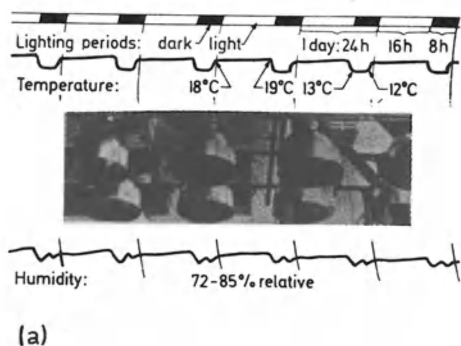
Pollen must be collected from freshly dehiscing anthers (Fig. 5d). This is best done about 1 h after the lights have been on in the morning. A sheet of aluminum foil is placed underneath the dehiscing spike which is loosely tapped. The pollen caught on the foil is transferred to a clean petri dish protected from light. Pollen should be used immediately after collection.

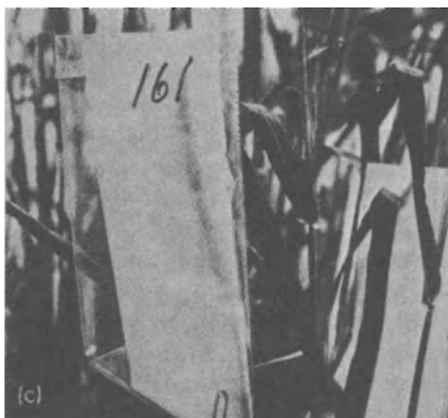
Figure 6 depicts some of the stages of monoploid production from growing the plants for the crosses to fruit production.

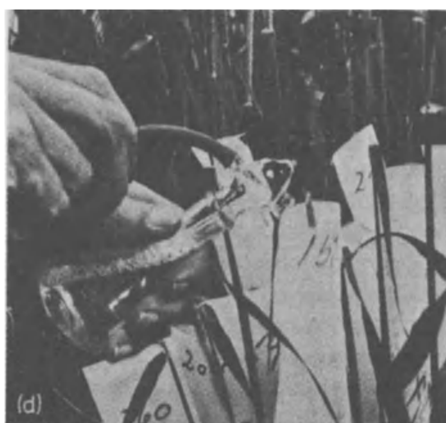
8.5 Pollination

Pollen is applied carefully and amply to the barley stigmas by: (a) dipping a sharpened toothpick into the pollen and applying it to the stigma, (b) dipping a fine camel hair brush into the pollen and lightly flicking it into the stigma region, (c) blowing the pollen on to the stigma with an aspirator.

The optimum stage of pollination depends on the time at which the flower is emasculated, temperature and genotype. Under Risø conditions and with Northern European barley cultivars, late emasculation can be followed by pollination the next day. But normally, the spikes are pollinated two days after emasculation. Pollen tube growth of *H. bulbosum* appears to proceed as normally as that of *H. vulgare* pollen on *H. vulgare* stigmas.







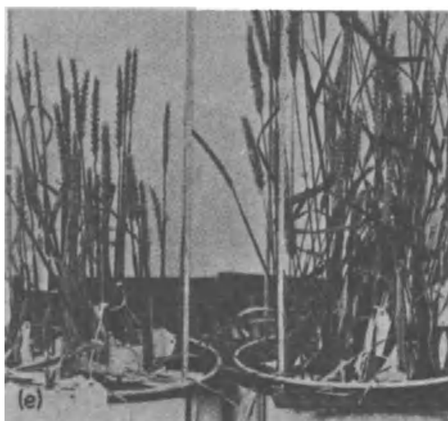


Fig. 6a–f. Stages of monoploid production: (a) Light regime and sources (HQIL - Osram - 400 W) and humidity for the growth of *Hordeum*. (b) Section of growth room with barley at flowering. (c) Enclosed spike of emasculated and pollinated in glassine and parchment isolation bag. (d) Spraying spikes with hormones. (e) Arrangement of cut shoots of barley in water culture (see text), *left*: pollinated spikes of barley, *right*: *H. bulbosum* and barley shoots mixed for pollination. (f) *Right*: Barley spike, with 15 florets with fruits from pollinations with *H. bulbosum*, 6 florets (bottom of spike) showing selfpollinated fruits (more plump due to presence of endosperm). (Spike 14 days after pollination.) *Left*: Petri dish with harvested fruits ready for sterilization

8.6 Hormone Treatment of Pollinated Spikes

Hormone treatment of pollinated spikes from one or two days after crossing does improve fruit set (LARTER and CHAUBEY, 1965; KASHA, 1974a; JENSEN, 1974a). The application should be repeated once or twice and the isolation bags left on the spikes to prevent the florets from drying out. Hormones may be applied to the florets by brush, syringe or by a fine spray as depicted in Figure 6d.

It has also been suggested that the hormone can be sprayed onto the foliage of the plant rather than the single spike, and a single application of 75 mg/l of gibberellic acid applied two days after pollination was found to be effective (SUBRAHMANYAM and KASHA, 1973). But applying the hormone repeatedly after pollination is probably more effective. Kinetin (ISLAM and SPARROW, 1974) may have a similar effect. JENSEN (unpublished) experimented with a number of hormones in mixtures (25 mg/l GA_3, 10 mg/l 6-benzylamino purine = BAP; 10 mg/l indole-3-acetic acid = IAA, 15 mg/l naphthalene acetic acid = NAA) and found no noticeable difference in fruit set; but embryo formation was advanced in comparison with that of GA_3 alone.

Morphactin, 2,3,5 iodobenzoic acid-TIBA (50 mg/l) in combination with cytokinin 6(γ,γ-dimethyl-allylamino)-purine = 2iP or kinetin at 10–20 mg/l and GA_3 (25 mg/l) and NAA (25 mg/l) was beneficial to rapid fruit development but with a large amount of liquid endosperm. This increased the chances of damage and embryo infection. The embryos, though, were comparatively large and developed well in culture. There was also an indication that spraying the hormone mixture more than once enhanced the development of the fruits. More work on these lines is needed before definite conclusions can be drawn.

8.7 The Cut-Shoot or Detached Tiller Method

At the time of or just before emasculation, shoots are detached at the base and placed in a water culture solution (modified Hoagland solution, see Appendix 2). It is advisable to burn about 3 cm of the cut end of the shoot base. This allows shoots from field-grown barley to be handled under environmentally controlled conditions and also provides for controlled nutrient feeding to spikes (JENSEN, 1973). One container can be used for about 60–70 shoots held in place by a perforated top of plastic or styrofoam (Fig. 6e). Air from the bottom of the container stirs and aerates the solution which is changed weekly. A plastic bag held by a support enclosing all spikes of a container will retain a high humidity level. Pollination is done by hand or by placing shoots of *H. bulbosum* about to shed their pollen beside and above the emasculated spikes of barley. The air stirring the water culture solution is enough to vibrate the spikes of *H. bulbosum* to ensure pollen release. Hormone treatment may be applied via the culture solution by adding 20 mg/l of gibberellic acid (GA_3) on the second day after pollination.

Table 1 shows that cut shoot and nutrient-cultured spikes produce as many embryos and monoploids as undetached shoots (see Sect. 11.2.6).

8.8 Harvest and Handling of Fruits

The optimum stage of harvesting the fruits for embryo culture varies with conditions and genotype. Embryos left too long in the fruits may show brown spots or become infected. Fruits which are dried up are difficult to dissect. There is a considerable variation in optimum harvest time due to: (a) Differences in nutrition of the embryos and fruits on the spike, (b) Fruit position on the spike in relation to distance from the artificial light source, (c) Genotypic differences in fruit and embryo development.

In practice the optimum stage for dissection is reached at 13–15 days after pollination when grown at +23° C (conditions for Guelph) and about 18–21 days when grown at +18° C (conditions for Risø).

For harvesting, the florets are pealed off the spike and care is taken to avoid damage to the fruit coat (Fig. 6f). Palea and lemma are removed and the intact fruit placed in a petri dish. The fruits are sterilized for five min. in a 5% calcium hypochlorite solutions plus a drop of Tween-20 or -80. Sterilization is followed by five consecutive washings with sterile water.

If not used immediately, the fruits may be stored at + 5° C in a sterile dish on filter paper moistened with culture medium (see Sect. 9). When stored thus for one week no reduction in viable embryos could be found.

9. Embryo Culture

9.1 Embryo Dissection

Instruments for dissection can readily be made following the descriptions given by CUTTER (1967), and ROMBERGER (1966). Dissection of embryos from sterilized fruits is done under 10× magnification in a stream of sterile air on a laminar flow bench. The fruits are placed in a sterile cover of a petri dish and slit open at the side with a dissecting needle to expose the liquid contents of the fruits. The embryo, often freely suspended in this liquid, is lifted out by a second needle and placed on the culture medium. Care should be taken not to damage the embryo and to place it with the ventral surface on the medium (NORSTOG, 1965). Callus growth can often ensue if the embryo is orientated upside down on the medium.

9.2 Media

Stock solutions should be prepared as 10× or 100× strength of the final concentration and stored cold (5° C). A stock solution can be checked by testing it against a standard cultivar (e.g. performance of 3-week-old Bomi embryos).

Three main media i.e. B_5 (GAMBORG *et al.*, 1968), B^{II} (NORSTOG, 1973), and R-M-IS (ISLAM *et al.*, 1974) have been used as agar solidified media. At Risø we have had more success with a floating culture system using a liquid medium (Appendix 1).

This system has one disadvantage in requiring the transfer of the small developing plantlets to an agar solidified medium for good root and shoot growth. It should eventually be possible to eliminate reculturing.

Liquid Media. Following the formula in Appendix 1, C-17 and C-21, the stock solutions are mixed, water added, pH adjusted and sterile filtered via a membrane filter unit as shown in Figure 7b (e.g. Millipore filter-type: GS 0.22 μ, filter holder type: XX4304700). The sterile medium can be stored in the cold. For the floating embryo technique (JENSEN, in preparation) about 8 ml of medium are put in 5 cm diameter sterile plastic dishes, a sterile millipore filter is floated on top of the medium. The excised embryos are placed on these filters and the dishes are sealed with strips of parafilm (See Fig. 7d).

Solid (Agar) Media. Appendix 1 gives the formula for the medium B_5 (see also GAMBORG *et al.*, 1968; KAO and KASHA, 1969). After mixing stock solutions and agar the medium is boiled to dissolve and the pH adjusted to 5.5. Glass vials are filled to one third of their capacity with medium, loosely capped and autoclaved in bulk. The autoclaved, sterilized medium can also be dispensed via a sterile funnel into sterile glass or plastic tubes or dishes.

9.3 Variation in Number of Embryos per Spike and Embryo Size

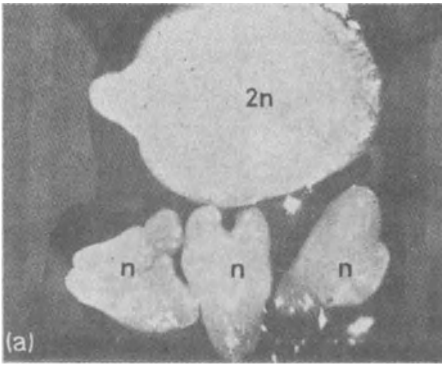
Often, in a production of monoploids, some spikes of a plant do not develop many fruits following these crosses with *H. bulbosum*. In most cases the first three to four formed shoots have the best fruit-setting ability. Fruits not properly formed rarely have a culturable embryo. Occasionally even well-formed fruits have no embryo. However, up till now we have no explanation for these shortcomings.

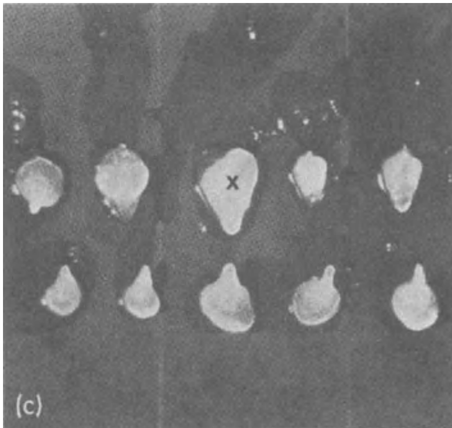
The embryos from the *H. vulgare* × *H. bulbosum* crosses grow and develop differently than those from normal diploid barley (Fig. 7a). They are monoploids and have a slower mitotic cycle (analogous to differences in mitotic cycle as described by BENNETT *et al.*, 1973); their nutrition during in situ growth is different since reduced endosperm formation disrupts normal nutrition.

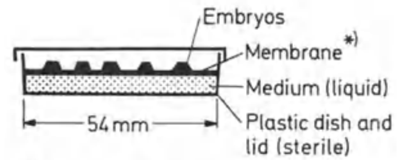
9.4 Culture Conditions

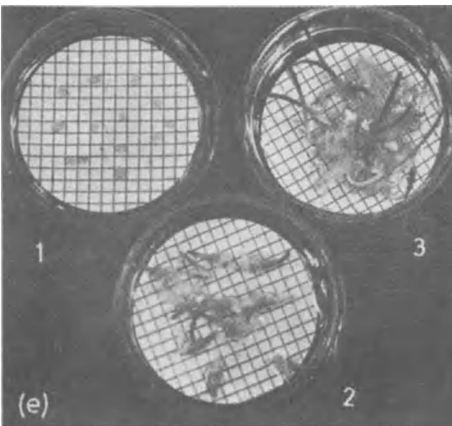
A culture cabinet with low light intensity (500–1000 lux) and temperature control from 15° C–25° C within a differential of ±1° C is adequate for embryo culture. A useful and relatively inexpensive growth cabinet has been described by DAVIES (1973). The first phase of culturing for one or two weeks is done in the dark at 18° C. Light encourages precocious germination. When properly differentiated (Fig. 7e, 2) the embryos can be moved into a 12 h light regime. At this stage a temperature of 20° C is used at Risø and 22° C at Guelph. When differentiated (Fig. 7e, 3) the embryos are moved from liquid to solid medium where they develop into plants with good roots within one or two weeks.

Once plants are established (Fig. 7f), the vials are transferred to growth room conditions with higher (about 40×10^3 to 50×10^3 lux) light intensities.









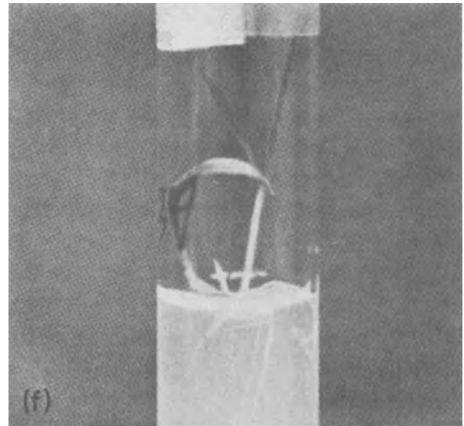


Fig. 7a–f. Stages in monoploid production: (a) 4 embryos of barley 14 days old from the same spike. $2n$ = selfpollinated, diploid embryo; n = monoploid embryos. $2n$ embryo measures 2.7 mm in length. (b) Arrangement for sterilizing liquid culture medium using a modified pressure cooker vessel, a glass beaker, a filter holder and Erlenmeyer flask for the collection of the medium which is forced through a sterile 0.22 μ filter using compressed air (see RIDDLE, 1973). (c) Ten 14-day-old embryos from the same spike following crosses with *H. bulbosum* on barley. All except X are monoploid. X is a hybrid and measures 1.6 mm in length. (d) Floating embryo culture: sketch of method used at Risø. (e) Three dishes of floating embryo culture with lids removed: *1* at start of culture, *2* 18 day of culture, *3* 25 days old culture. (f) Monoploid sporophyte in culture tube

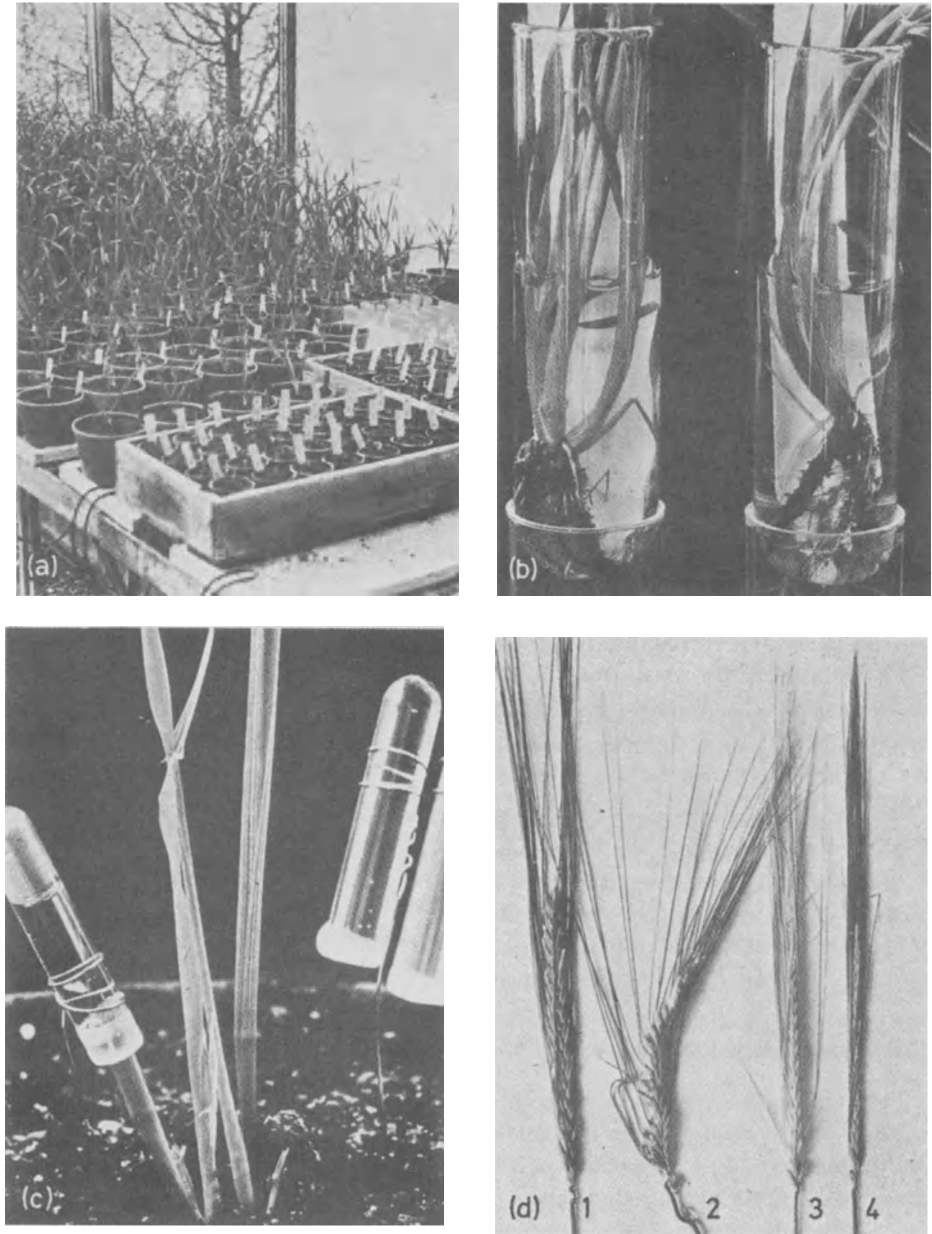


Fig. 8a–d. Schematic view for chromosome doubling: (a) Growing monoploids into sturdy plants. (b) Treating shoot-plants with colchicine solution plus a carrier (DMSO). (c) Treatment of a monoploid plant with colchicine via the capping method (inverted vial). (d) Different categories of spikes on a colchicine treated monoploid; *1* almost completely fertile spike (bearing chromosome doubled embryos); *2* a chimeric spike, one half with normal seeds (doubled), the other half with no seed set (monoploid, non-doubled); *3* and *4* typical spikes with monoploid character, sterile florets

10. Plant Culture and Multiplication

10.1 Transfer to Soil Culture

Plantlets cultured in vitro require hardening and good root development to cope with transplantation to the soil. About a week before transplanting the culture vials are placed in a greenhouse and receive about 15×10^3 lux of light at 20° C 18 h light, 12° C 6 h dark.

They are lifted carefully with some agar on the roots to small clay pots filled with a light soil mixture (sand:peat:soil in 1:1:1 by volume). The potted plants are put in a tray with peat and covered by a plastic tent to prevent desiccation. Growth commences from a few days to a week later and the plants can be adjusted to ordinary greenhouse conditions. Care must be taken not to overwater the freshly potted plants and to protect them from desiccation. The balance between dry and moist conditions results in a good survival of plants (Fig. 8a).

10.2 Screening Plants for Hybrids and Off-Types

When the plants have grown to the two to three tiller stage the following grouping can be made on morphological characters:

Barley Monoploids. Upright shoots, narrow leaves, no visible hair, slender but vigorous in tillering.

Hybrids. Type 1: Prostrate growth habit, leaves slightly curled and dark green, visible hair on leaves, excessive tillering. Type 2: Upright growth, foliage slightly broader than monoploids, hair distinctly visible on leaves and stems.

Off-Types. (Includes aneuploids, triploids, chimeras etc.). Plants very irregular, a range of intermediate types from between previous two groups.

Before chromosome doubling the material is screened for monoploids. This morphological grouping has been found to be reasonably accurate. Possible hybrid plants need not be discarded as the *H. bulbosum* spike characters seem to dominate over the *H. vulgare* characters.

10.3 Chromosome Doubling

Vigorous plants at the three to four tiller stage are treated as follows: Soil is washed off the roots, which are cut back to about 3 cm below the crown. The plants are placed in a glass vial (Fig. 8b). The colchicine solution (2.5 g colchicine dissolved in 20 ml dimethyl sulfoxide (DMSO) is made up to 1 litre with water. A few drops of Tween-20 or -80 are added. Enough of this solution is placed in the vials to cover the plant crowns and part of the leaves. (See Sect. II.2.7 for plant stages and colchicine dosage).

The plants under treatment (Fig. 8b) are placed in a growth chamber (+20° C in the light) for 5 h, and potted directly after treatment into a light soil.

At Guelph plants are treated whilst still in the culture vial. The method is essentially the same except for a washing and temperature treatment at + 32° C for three hafter colchicine application.

Plants not responding to this treatment may be cut back and treated by the inverted vial method (see JENSEN, 1974b and Fig. 8c). At Adelaide this tiller-capping method (on plants with 2–3 tillers) has proved highly successful for chromosome doubling (ISLAM and SPARROW, 1974).

10.4 Rogueing and Seed Harvesting of Doubled Monoploids

Given optimum conditions for flowering the colchicine treated plants flower from one to two months later. The first formed shoots are usually monoploid (sterile and two third the size of normal diploids). Later formed shoots bear from a few seeds per spike to almost full seed set (Fig. 8d). Cross pollination is negligible and thus isolation (by bagging) of the spikes is not necessary.

During flowering and seed harvest the material should be rogued for abnormal spike and other plant characters.

After harvesting, the seed is sown for multiplication and again a further screening for off-types is practised. Tetraploids and doubled monoploids are distinguished either by their broader leaf shape, darker color, growth habit or irregular flowering. When sown in the field as lines the doubled monoploids are found very uniform in plant character within the respective lines demonstrating their homozygosity.

11. Discussion and Remarks

11.1 General

The problems of producing monoploids and doubled monoploids in barley are mainly technical. The advantage of anther and microspore culture techniques are obvious, but extensive research is needed before these methods can be as efficient as the Bulbosum method. Recent research in microspore methods (NITSCH, 1974a, b, 1975; REINERT *et al.*, 1975; Bajaj *et al.*, 1975) points to advances with simple, technical adjustments of procedures (e.g. preconditioning anthers in situ, centrifugation and cold shock treatment, washing of microspores and addition of amino acids promoting embryogenesis). There is also the possibility of separation and selection of viable microspores as for rye (WENZEL *et al.*, 1975) and the induction in situ of a type of microspore prone to form embryos (SUNDERLAND, 1974). It has been observed that in vitro culture of barley cells and tissues is often carried out on media with too high a concentration of solutes. Decreasing the levels of solutes by one half of the standard media and adjusting the osmolarity with mannitol gives better organogenesis (JENSEN, unpublished).

Experience has shown that the Bulbosum method is highly reproducible on almost any barley material even though conditions for growing and treatment are not identical.

The method of hybridization followed by chromosome elimination proves to be of general interest for haploid induction in other species of *Hordeum* (RAJHATHY and SYMKO, 1974) and also of cultivated hexaploid wheat, *Triticum aestivum*

(BARCLAY, 1975). These recent reports no doubt will contribute to a broader interest in this method. At the same time work on the chromosome elimination mechanisms (DAVIES, 1974; KASHA, 1974a, SAGAR and KITCHIN, 1975) will become of high priority with a view to utilize haploid induction in general.

11.2 The Bulbosum Technique

11.2.1 Growing Conditions

The following discussion concerns the problems of the Bulbosum technique and suggestions for its improvement. The importance of the growing conditions for barley have been stressed (JENSEN, 1974a; KASHA, 1974a). Barley is photoperiod-sensitive and under suboptimal conditions short days can lead to sterility (BATCH and MORGAN, 1974).

Work on vegetative and reproductive growth phases under controlled environmental conditions (phytotron studies by DORMLING *et al.*, 1975) show that even in related genotypes of barley the plants express themselves differently under similar conditions. These studies point to the need for more detailed studies on optimum growing conditions of the genotypes for monoploid induction. Also the nutrition of the plant should not be neglected (see HEWITT, 1966; MACLEOD and CARSON, 1969).

11.2.2 *Hordeum bulbosum* and Pollen

Handling *H. bulbosum* plants for pollen production usually causes no problems when well developed bulbs are available. Studies on the reproductive development of tetraploid *H. bulbosum*, indicate that the photoperiod as well as vernalization at low temperatures determine phase changes (KOLLER and HIGHKIN, 1960). Storage of *H. bulbosum* pollen is difficult, so that a continuous supply of pollen-producing plants must be available during crossing. As indicated before, *H. bulbosum* as on outcrossing species is variable in terms of visible morphological characters. Even though we have made our observations on comparatively limited material (less than 100 genotypes) it seems types adapted to our growing conditions and pollen-producing requirements can be selected.

11.2.3 Hybridization

In crossing work, fruit induction and embryo development on the spike the following comments can be made: emasculation, although costly, cannot be neglected and methods to increase the efficiency of emasculation should not damage the stigma. Scissors emasculation is more expedient than sideways emasculation but tends to give a lower fruit set. (For a discussion of emasculation techniques see: POPE, 1944; HAMILTON, 1953; WELLS and CAFFEY, 1956). Some cultivars of barley are sensitive to mechanical damage to the flower which might result in complete female sterility (QUALSET and SCHALLER, 1968).

Low humidity perhaps more than anything else, in the period between emasculation to the embryo development is the main cause of reduced fruit set. Even a

relative humidity of 80% in the crossing area may be insufficient to prevent the desiccation of emasculated flowers. The type of isolation bag may also be critical in obtaining good fruit set.

11.2.4 Fruit Induction and Ovular Growth

Very little work has been done on fruit induction following difficult crosses or abnormal fertilization in barley. The classical work of VAN OVERBEEK *et al.* (1941) on chemical stimulation of ovule development has had no success with Gramineae. In situ experiments by JUNGFER (1952) in barley have been negative. In vitro experiments in culturing ovaries or ovular structures of barley have been unsuccessful (WALKER and DIETRICH, 1963; KLASSEN and LARTER, 1967). A study of the ovular sap under embryo development led SMITH (1973) to compose an analytical culture medium—i.e. one that was of similar composition as the analysis of the sap had shown. His interesting approach will be applied to our barley material.

Normal embryo development is tightly linked to development of the flower (LA CROIX *et al.*, 1962). Treating fertilized flowers with different hormones (Gibberellic acid, indole-3-acetic acid, naphthalene acetic acid), alone or in combination with cytokinins, does not seem to be effective. One exception, however, was found at Risø when spraying with a mixture of GA_3, cytokinin, and morphactin. Here, larger embryos resulted and harvesting of fruits for embryo dissection could be delayed by almost one week without a decline in plant production.

11.2.5 Endosperm Development and Nutritional Aspects

An interesting case in endosperm/embryo relationship is found in barley. Here mutants are known that either develop only embryos (without any endosperm) or endosperm without any embryo development (HARLAN and POPE, 1925). At Risø we have recently found a mutant with no endosperm formation but with embryo development. This type has been termed "watery kernel" (HARLAN and POPE, 1925), and for perpetuation it has to be excised from the fruit about two to three weeks after pollination or else it dies. Its embryos grow readily on any of the media listed in Appendix 1. Besides being of use in studies indicated by RICE and CARLSON (1975) the endosperm-less diploid mutant will be used in a comparative study on embryo development in barley in conjunction with the monoploid embryo from interspecific crosses. A better understanding of nutritional requirements for the embryo might be obtained by studying this mutant.

RUTISHAUSER (1969) has provided a detailed analysis of genome balances in different cross combinations and the different endosperm genomic make-ups. On similar grounds a tabulation is given for the different crosses of cytotypes of *H. vulgare* and *H. bulbosum* (see Fig. 9). The fate of the endosperm development in these interspecific hybrids has not been fully examined although there are indications (SUBRAHMANYAM and KASHA, 1973) that it may be similar to barley × rye crosses (ODENBACH, 1965) and wheat × rye crosses (MOSS, 1972; BRINK and COOPER, 1947).

Observations at Risø indicate, however, that the decline in endosperm development is abrupt and varies in time due to genotype combination rather than to

GAMETE COMBINATION ♀	♂	EXPECTED GENOMIC CONSTITUTION EMBRYO SPOROPHYTE	ENDOSPERM
V	B	V	(VVB)-
B	V	V	(BBV)⊗
V	BB	VBB	(VVBB)-
BB	V	BBV	BBBBV+
VV	BB	VV	(VVVVBB)-
BB	VV	VV	(BBBBVV)×

Fig. 9. Possible cross combinations of various cytotypes of *H. vulgare* and *H. bulbosum*. The combinations are listed as genomes; *V* one *H. vulgare* genome; *B* one *H. bulbosum* genome. Endosperm formation: + normal; − endosperm disintegrates; × endosperm formation not always stable. (After data of SUBRAHMANYAM and KASHA, 1973)

environmental conditions. This leads to the question of how to influence chromosome elimination in the embryos without disrupting "normal" development of the endosperm.

11.2.6 Zygote Formation and Embryo Development in situ

After pollination with *H. bulbosum*, fertilization in barley seems to proceed in a way similar to that described for selfed barley (POPE, 1943; LUXOVA, 1967; CASS and JENSEN, 1970; and W.A. JENSEN, 1973). However, after zygote formation and central cell fertilization a different set of conditions operate (SUBRAHMANYAM and KASHA, 1973). At times more than one monoploid embryo develops in these fruits. Several times we have extracted three embryos from a single ovule, giving viable monoploid plants. The origin of these extra embryos is not known. As Figure 5c shows, there are several possibilities for extra embryo sac-derived embryos from synergids, central cell or antipodals. Under conditions at Risø, Bomi (a cultivar of *H. vulgare*) × *H. bulbosum* embryos have 10, 19, and 242 cells after two, three or six days of pollination, respectively. This corresponds roughly to the figures given by SUBRAHMANYAM and KASHA (1973). It is not known whether the differences in environmental conditions, the genotype of barley or of *H. bulbosum*, or all factors together determining the rate of chromosome elimination. Certainly, temperature is a decisive factor in rate of embryo growth.

Observations show that the rate and mode of development for the monoploid embryo and selfed diploid barley embryo differ, i.e. monoploid embryos have cells smaller than those of diploids, their mitotic cycle and rate of growth is also comparatively slower. Morphologically the monoploids differ from normal diploids in shape, especially the scutellum which often has an irregular heart-shaped form in monoploids (Fig. 7a). Very often the suspensor is not as prominent in monoploid embryos as in normal diploids. Selfed diploids of the endosperm-lacking mutant (see Sect. 7.3.5), on the other hand have a shape very similar to monoploids yet are somewhat larger. NORSTOG (1972a) has described the early development of selfed diploid barley embryos; and has subsequently shown

Table 1. Comparison between greenhouse and field-grown barley when treated as detached and normal shoots in monoploid production

Location	Spike treated	Florets pollinated	Fruits harvested	Embryos cultured	Monoploids obtained	Monoploids as % of embryos
Field	Detached	456	388	171	59	34.5
Field	On plant	392	121	42	10	23.7
Glasshouse	Detached	422	359	182	98	53.8
Glasshouse	On plant	430	371	169	85	50.3

(NORSTOG, 1972b, 1974a) that the presence of a suspensor, which firmly attaches the developing embryo to the ovular tissues, restricts early embryo dissection. Monoploid embryos, often even at very early stages of development, appear to float in the sap of the fruits on dissection. As mentioned in various reviews (NORSTOG, 1972b; KAPIL, 1974; DURE, 1975) little is known about the mechanism of nutrition of the embryo and ovular tissues. In barley KIRBY and RYMER (1974) have described the vascular system supplying the single florets. Much more work is needed to understand and find ways of influencing the nutrition of embryos in situ. A comparison has been made (Table 1) between cut-shoot and plant developed embryos, both from field material and under glasshouse conditions.

The results of detached and non-detached shoot treatment are not consistent for the different cultivars. It can be concluded, however, that in general practice there is little difference between the two methods. The advantage of the detached method is that field-grown material can be brought indoors, and the simultaneous treatment of large numbers of spikes with pollen and hormones is possible.

The best time of harvesting the fruit seems to vary with type of cultivar and growing conditions. (At Guelph the optimum time is about two weeks after pollination, whilst at Risø it is some five to seven days later.)

11.2.7 Embryo Culture, Genotypic Effects, and Chromosome Doubling

Diploid Hordeum vulgare Embryos. The main emphasis in application of embryo culture in genetics and plant breeding has always been in helping to raise plants from embryos developed under incomplete endosperm development (MAHESHWARI and RANGASWAMY, 1965). Work by NORSTOG (1965, 1970, 1972b, 1973) concentrates mainly on developmental aspects of the barley embryo per se. However, diploid embryos of barley have different culture requirements for optimum growth than monoploid embryos.

Monoploid H. vulgare Embryos. The media listed in Appendix 1 give good results with embryo cultures and especially medium C-17 supports substantial growth of both monoploid and diploid embryos.

Table 2 shows the results of embryo growth and plant production on agar-solidified liquid medium C-17. There are clear advantages of using the liquid form as both genotypes have performed better on the liquid culture media than on the solid. In our experience irregularly developed small embryos have a better chance of growth on liquid than on agar media.

Table 2. Growth of monoploid embryos on liquid and agar-solidified C-17 medium. Embryos from HP40 and F_1 (HP40 × 1508) after crossing with *H. bulbosum*

Medium	Cultivar	Number of embryos cultured	Plants from embryos		% of cultures with:		
			Number	%	Roots only	Shoots only	Callus only
Liquid	HP 40	242	108	44.6	9.1	5.3	8.4
Solid	HP 40	245	67	27.3	7.5	8.7	15.6
Liquid	F_1	231	129	55.8	7.3	7.5	10.9
Solid	F_1	236	72	30.5	6.2	11.2	18.3

Table 3. Embryo culture and plant production in an F_1 (HP40 × 1508) compared to 1508 and HP40 as the parents

Combination or cultivar	Number of					Monoploid		Total plants	
	Spikes	Flowers	Fruits	Embryos	Embryos per fruit %	Plants	Plants % of embryos	No.	Embryos %
HP 40	24	415	372	224	60.2	89	39.7	106	47.3
1508	25	412	325	170	52.3	53	31.2	69	40.6
F_1(HP40 × 1508)	23	420	403	312	77.4	196	62.8	218	69.9

Washing the freshly dissected embryos in the medium before transfer to the culture dish has resulted in increased development (JENSEN, in preparation), however it is not clear whether washing removes some inhibitor from the embryo. In some instances dormancy can set in, even in not fully developed embryos (see also RYCZKOWSKI, 1971).

Despite the many steps in technique of monoploid production (Fig. 4a) it has been possible at Guelph, Risø, Aberystwyth, Cambridge and Adelaide to produce monoploids by this method at ever increasing frequencies. By our continued experience with this technique we have increased our production from 5 or 6% monoploids per embryos cultured in 1971/1972 to about 62% of success in 1973/1974, which is very encouraging.

Genotypic Effects on Monoploid Production. The differential behavior of genotypes as exemplified in Table 3 affects the success of embryo culture. Here, the F_1 between two cultivars (HP40 × 1508) gives consistently higher yields of monoploids than the parent varieties. There is also a great variation within cultivars. Whether these differences are determined genetically or are purely an expression of the physiological status of the donor plant at the time of treatment or of the embryo per se is not known.

We also have evidence that the pollen parent, *H. bulbosum*, influences the development of embryos and determines in part whether chromosomes are eliminated completely or whether hybrids result. This interaction of *H. vulgare* and

Table 4. Response of different barley genotypes in crosses with a clone of *H. bulbosum* presented as percentages of monoploids and hybrids

Genotype		Embryos cultured	Plants					
H. vulgare ♀	*H. bulbosum* ♂		Total	% of embryos	Monoploids		Hybrids	
						% of embryos		% of embryos
HP40	102	288	159	55.2	130	45.1	29	10.1
Bomi	102	257	145	56.4	138	53.7	7	2.7
Sultan	102	270	159	58.9	118	43.7	41	5.2
Mutant 1508	102	172	83	48.3	75	43.6	8	4.7

H. bulbosum in determining frequency of monoploids and of hybrids, needs further experimentation.

Table 4 shows the influence of the female genotype (here *H. vulgare*) and *H. bulbosum* on hybrid frequencies (i.e. incomplete chromosome elimination). The cultivar Sultan shows a much higher frequency of hybrids than the other cultivars tested. There is some evidence that embryos can be graded at dissection into hybrid and monoploid embryos. Although high frequencies of hybrids in a production series mean reduction in number of monoploids, the screening of hybrids is a comparatively easy task as the morphological differences of hybrids and monoploids are very distinct.

Precocious Development in vitro. Immature embryos when cultured in vitro do not normally go through all stages of embryogenesis to become dormant, but germinate precociously (NORSTOG and KLEIN, 1972; NORSTOG, 1972b). This means that the less an embryo is developed at the time of excision the more likely it is that a weak, underdeveloped plantlet is obtained. To get viable plantlets from immature embryos means culturing them into mature embryos and it seems that our liquid medium C-17 is favorable for prolonged embryo growth.

From in vitro Culture to Soil Culture and Chromosome Doubling. Transfer of monoploid plants from in vitro culture systems to soil and glasshouse growing conditions requires sturdy plants, a well aerated soil and the right amount of moisture.

Emphasis should be placed on treating the material uniformly on a rational scale. The sooner the plants are established and ready for colchicine treatment the quicker the homozygous seed will be set. Doubling treatments commence when three to five shoots have been produced on the vigorously growing monoploids. Depending on the size, age and state of the monoploids various concentrations of colchicine dissolved in 2% dimethyl sulfoxide can be administrated for a period of up to five hours in the culture tube, or in petri dishes 0.01–0.05% for plants with 2–3 shoots, 0.05–0.1% for plants with 3–5 shoots, 0.2–0.5% for 4–12-week old plants. Following this treatment the plants must receive good growing conditions to stimulate vigorous growth. On average about one half of the treated shoot initials will double and consequently set seed. An encouraging observation

is that the chromosome doubling can be made efficient by relatively small changes in treatment. As WALSH *et al.* (1973) have pointed out, a possible seed source effect is circumvented by increasing seed from a doubled monoploid in a further generation and using this in replicated field trials.

A proposal has been made (JENSEN, 1974b) to term the various generations after monoploid induction. At the same time it was pointed out that different ploidy levels can arise directly after colchicine application and for this reason any plants other than diploid can easily be screened off.

11.3 The Process of Monoploid Induction, Chromosome Elimination and Somatic Reduction

11.3.1 The Bulbosum Process of Chromosome Elimination

The process by which monoploids via the Bulbosum method in barley are obtained is one of somatic chromosome elimination of the *H. bulbosum* parent, however the mechanism of preferential chromosome elimination is not fully understood. KASHA (1974a) has reviewed this subject, and HO and KASHA (1975) have reported on the genetic control of the mechanism. Using a test system of a trisomic series in *H. vulgare*, crossing with tetraploid *H. bulbosum* and finally testing with telotrisomics of chromosome 2 and 3 revealed that the genes for elimination are most likely situated in 3L, and also that some genetic factors are found in both arms of chromosome 2. Cytologically, the process of elimination is a gradual one. Elimination is most prominent in actively dividing tissue such as embryos, young endosperm or meristems. Three possible hypotheses for the elimination mechanism have been proposed (KASHA, 1974a). (1) Asynchrony of mitotic cell cycle times due to differences between the potential species. (2) Spindle or centriole abnormalities. (3) A system similar to "modification-restriction" in bacteria and bacteriophage.

The first system would work in much the same way as in mammalian cell hybrids grown in culture (KAO and PUCK, 1975). Here the preferential elimination works by cell cycle times, the type with the slower cell cycle time tending to be eliminated (SUBRAHMANYAM and KASHA, 1973). This system has also been proposed for chromosome elimination in certain *Nicotiana* hybrids (GUPTA and GUPTA, 1973).

The second system involves spindle or centromere attachment problems as described for example in certain insects (CAMENZIND, 1974). In this connection it is worth pointing out that barley sperm cells reach the 2C condition (D'AMATO *et al.*, 1965) before pollen dehiscence and the sperm cells are probably at the G2 stage when fertilization takes place. If this is the same for *H. bulbosum* the argument is against this system as only stages after the S-phase would affect elimination of zygotic chromosomes.

The third possibility has been proposed by DAVIES (1974). In this system foreign DNA is recognized by a nuclease compound and can then be inactivated. SAGAR and KITCHIN (1975) in a recent account have treated this subject in detail bringing forth their results of selective destruction of DNA in *Chlamydomonas* and explained chromosome elimination in other plant and animal systems.

11.3.2 Chromosome Elimination and the Influence of Parent Genotypes, Species and Other Genera

Whatever the systems involved, the application of it points to a stable mechanism. Recently it has been observed that monoploid and especially hybrid frequencies in *H. vulgare* × *H. bulbosum* crosses depend on the genotype of both parents (Table 4). Work is in progress to try and isolate *H. bulbosum* genotypes which induce high frequencies of monoploids and also those which give high frequencies of hybrids which in turn can be used in gene transfer programs. Likewise, recent experiments at Cambridge (FINCH, personal communication) and at Risø indicate that the genotype of *H. vulgare* in combination with *H. bulbosum* genotypes determines the rate and efficiency of chromosome elimination.

The elimination of chromosomes is not restricted to *H. vulgare* × *H. bulbosum* crosses but seems to be widespread in *Hordeum* species (ISLAM and SPARROW, 1974; KASHA, 1974a; RAJHATHY and SYMKO, 1974). In this way haploids have been produced in interspecific and intergeneric crosses between *Hordeum* species (PRICE, 1968; AHOKAS, 1970; KASHA, 1974a).

Recently, wheat haploids have been produced in large frequencies by crossing wheat with pollen of *H. bulbosum* (BARCLAY, 1975). The system requires embryo culture and works essentially on experimental procedures similar to the production of barley monoploids by the Bulbosum method.

11.3.3 Consequence of the Bulbosum Method of Somatic Chromosome Reduction

As illustrated in Figure 9, as a general rule, the chromosome elimination process depends partly on a genome balance. Mainly for this reason endosperm formation can be ruled out in systems where a diploid barley is used as the female parent and a diploid *H. bulbosum* as the male parent.

Another irregularity is the malformation of the antipodal cells which often become highly polyploid before disintegrating. What is important to stress, however, is that since embryo induction is obtained in high frequencies, a random sample of gametes can be expected from this system of monoploid induction.

Another question concerning the formation of monoploids by the Bulbosum method is that there might be a chance during fertilization and early zygotic stages for *H. bulbosum* material to influence the development of the *H. vulgare* monoploid. The evidence to-day is that although this chance does exist, it nevertheless does not manifest itself in the material from the monoploid productions. On the contrary, most attempts to transfer genetic material from *H. bulbosum* to *H. vulgare* have so far been unsuccessful.

CASS and KARAS (1975) have shown on an ultrastructural level that barley sperms (gametes) are totally naked and possess no mature plastids. Cytoplasmic transfer from *H. bulbosum*, supposing it has a similarly formed sperm cell and fertilization proceeds normally, must therefore be unlikely.

Another interesting aspect of the preferential chromosome elimination process is that it is possible to produce monoploids of barley in a cytoplasm of

H. bulbosum by using *H. vulgare* as the male and *H. bulbosum* as the female. In this instance valuable material for cytoplasmic studies can be produced, again using embryo culture as a vehicle to obtain high frequencies of "foreign cytoplasm" monoploids.

11.3.4 Somatic Chromosome Elimination in Other Genera and by Other Processes

With regard to the process of somatic chromosome elimination information has been given by workers hybridizing somatic animal cells (EPHRUSSI, 1972). The ability to culture protoplasts of *H. vulgare* and of *H. bulbosum* followed by somatic hybridization would make an ideal system to study chromosome elimination. These studies could be supplemented by techniques to differentiate the chromosomes of the two species by banding patterns and the ability to regenerate from the fused somatic cells (see Chap. IV.1.2 of this Volume).
As already mentioned in Section 11.3.1. *Hordeum* species may not be the only one where chromosome elimination is found in higher plants, KASHA (1974a) has listed a number of other cases, JACKSON and JORDAN (1975) describe a possible case for *Haplopappus gracilis* where a monoploid has been examined cytologically with only two chromosomes.

Whether or not chemical agents can be used as efficiently to affect somatic chromosome reduction remains to be seen. KASHA (1974a) and ZENK (1974) give examples where chemicals such as: colchicine (in *Sorghum*); 3-fluorophenylalanine (in *Lolium* × *Festuca* hybrids) or parafluorophenylalanine (in *Nicotiana* cultures); chloramphenicol (in *Hordeum* root tips) have resulted in reduced chromosome numbers in the plant cells or tissues. However, both these authors conclude that somatic chromosome reduction via chemicals is not properly understood and should therefore not be viewed toó optimistically as a practical method of inducing haploidy.

12. Anther and Pollen Culture

The status on anther culture can be summed up as follows: CLAPHAM (1973) (see also Chap. II.3) obtained relatively high callus formation with cultivars Sabarbis and Akka or with various hybrids of barley cultivars. The anthers were cultured on medium B (essentially LINSMAIER and SKOOG, 1965) with high sucrose (12%) levels and various levels of antiauxins (0.02 mg/l tri-iodobenzoic acid, TIBA). Under favorable conditions 29.6% of anthers showed callusing. However, the number of plants produced were few and showed various ploidy; out of 25 plants three were predominantly haploid; one partly haploid, 17 diploid and four predominantly tetraploid.

These results were reproduced by MALEPŚZY and GRUNEWALDT (1974), who found in addition a genotype effect in that only one cultivar of the various genotype tested, "Amsel", yielded plants of various ploidy levels. PEARSON and NILAN (1975) also report a genotypic response, as the spring cultivars Akka, Zephyr, Unitan, Traill, and Trebi formed callus and even some plantlets. The

winter cultivar Hudson, on the other hand, did not produce callus or plantlets. One major handicap was that the plantlets produced were chlorophyll-deficient and could not be kept alive for long. The recent result of GRUNEWALDT and MALEPSZY (1975) point out the real difficulties of anther culture in barley, i.e. relatively high callus formation with differences between cultivars. In cultivar Vogelsanger Gold 32% of the anthers, yielded calli, however, even though about 1000 plantlets could be formed from three-week-old callus, almost all except four were chlorophyll-deficient, aneuploids and/or polyploids. ZENKTELER and MISIURA (1974) have also reported the formation of pollen embryos in barley.

Chlorophyll deficiency and different levels of ploidy make the plantlets from anther culture of little use in the production of monoploids in plant breeding and genetics.

Hopefully, the inspiring work on the pollen or microspore culture in Solanaceae species (NITSCH, 1974a) can one day be successfully extended to barley. WENZEL *et al.* (1975) have described an interesting technique of separating viable rye microspores from non-viable ones. They argue that the asynchronous development of microspores might play a role in diminishing the chances of microspores dividing.

13. Monoploids via Protoplasts

Work on the culture of protoplasts isolated from tetrads is still at the very beginning (BAJAJ, 1974). The potentials of using this scheme are obvious as monoploids obtained directly from the process of meiosis would have many advantages over those from later stages, e.g. early microspores (potential male gametes) (see

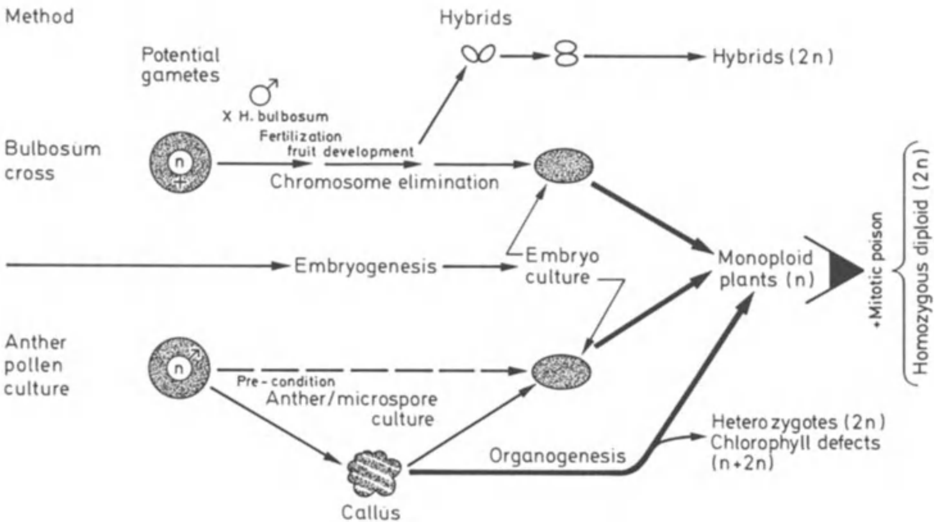


Fig. 10. Schematic representation showing possible routes of monoploid and doubled monoploid production

Fig. 10). KAO *et al.* (1974) have established a method for handling diploid barley protoplasts from leaves.

The system described here—the Bulbosum method, which is based on a gametic fusion followed by somatic chromosome elimination in the early embryonic cells should make an interesting subject for somatic cell fusion. For example protoplasts of monoploids of barley and *H. bulbosum* can be fused and by a chromosome banding procedure recently developed for barley (unpublished) the fate of the single chromosomes can be studied in these fusion products.

14. Retrospect and Prospect

For anther and pollen culture the main obstacle in barley seems to be the inability to obtain normal green plants with high frequency from induced callus. This lack of normal chlorophyll production seems to be common to many Graminae in vitro cultures (GAMBORG *et al.*, 1970; CLAPHAM, 1973; Chap. II.3). CLAMPHAM'S observations and the report by TULECKE (1967) indicate that alterations in the culture medium might bring about different frequencies of chlorophyll-deficient plants. It is noteworthy that plants regenerated from callus obtained from the scutellum of monoploid *H. vulgare* embryos were mostly with normal chlorophyll development (JENSEN, unpublished). There was no change in frequencies of normal green monoploid plants from this callus when cultured on other media than those listed in Appendix 1 (i.e. media of LAMPORT, 1963; SCHENK and HILDEBRANDT, 1972; KRUSE, 1974). This leads one to speculate whether the origin of the callus or the mode of its initiation may also play a role in chlorophyll-deficiency as found in barley.

The Bulbosum method depends in the first place on ability to hybridize and to induce high frequencies of embryos. *H. bulbosum* need not be the ideal partner for *H. vulgare* to induce monoploids of barley via somatic chromosome elimination. There can well be a range of *Hordeum* (KASHA, 1974a; PRICE, 1968) that might be tried as a more efficient partner than *H. bulbosum.* On the other hand, there seems to be much variation in different genotypes of *H. bulbosum* regarding ease of chromosome elimination. It is our aim to try and select for this variation in *H. bulbosum* collections.

Somatic chromosome reduction with the aim of inducing haploidy might also be achieved by applying, for example, halogenated amino acids. As yet no conclusion can be reached whether this methods is of practical value to induce high haploid frequencies.

The main advantage of the monoploid method is seen as a speeding-up of material for testing recombinants. Whether the monoploids for this purpose are derived from potential male or femal gametes is of no importance in barley. What is important is that normal monoploids can be produced in high frequencies.

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References see page 331.

Appendix 1. Composition of media used in embryo culture of monoploid barley

	Chemical	B5 mg/l	B^{II} mg/l	R-M-IS mg/l	C-17 mg/l	C-21 mg/l
Macro-nutrients	KNO_3	2500		150	300	300
	$CaCl_2 \cdot 2H_2O$	150	740		250	
	$MgSO_4 \cdot 7H_2O$	250	750		325	300
	$(NH_4)_2SO_4$	134				
	$NaH_2PO_4 \cdot H_2O$	150			100	
	KCl		750		150	300
	KH_2PO_4		910		150	500
	$Ca(NO_3)_2$			350		500
	NH_4NO_3				200	
Micro-nutrients	KI	0.75			0.10	
	H_3BO_3	3.0	0.5	0.5	5.0	15.0
	$MnSO_4 \cdot 4H_2O$	10.0	3.0	3.0	0.5	
	$ZnSO_4 \cdot 7H_2O$	2.0	0.5	0.5	0.25	
	$Na_2MoO_4 \cdot 2H_2O$	0.25	0.025	0.025	0.012	
	$CuSO_4 \cdot 5H_2O$	0.025	0.025	0.025	0.012	
	$CoCl_2 \cdot 6H_2O$	0.025	0.025	0.025	0.012	
	Na_2EDTA	37.3			·	
	$FeSO_4 \cdot 7H_2O$	27.8				
	Ferric citrate		10.0	10.0	3.0	20.0
	Fe EDTA				17.5	10.0
Vitamins	Nicotinic acid	1.0				
	Thiamine HCl	10.0	0.25	0.25	0.25	10.0
	Pyridoxine HCl	1.0	0.25	0.25	0.25	
	Inositol	100.0	50.0	50.0	50.0	150
	Ca-pantothenate		0.25	0.25	0.25	
	Glycine				0.75	
	L-ascorbic acid				0.50	
Amino acids	L-glutamine		400			
	L-glutamic acid			200	150	300
	L-alanine		50		30	
	L-cysteine		20			
	L-arginine		10		20	50
	L-leucine		10		10	
	L-phenylalanine		10		20	
	L-tyrosine		10			

Appendix 1. (continued)

	Chemical	B5 mg/l	B^{II} mg/l	R-M-IS mg/l	C-17 mg/l	C-21 mg/l
Amino acids	L-tryptophan		10			
	L-aspartic acid				30	100
	L-proline				50	50
	L-valine				10	
	L-lysine				10	
	L-serine				25	25
	L-threonine				10	
	Sucrose	20000	34200	20000	60000	45000
	Agar (DIFCO)	7500	6000[a]	7000	—	—
	pH	5.5	5.0	5.0	5.5	5.5

[a] Purified.

In addition: Media B^{II}, C-17 and C-21 have the following additives per/l medium:

B^{II}: Malic acid, 1 g dissolved in 50 ml H_2O, pH 5.0.

C-17: Citric acid, 500 mg in 50 ml H_2O, pH 5.3. tri-potassium citrate, 300 mg, added to final medium, pH of medium adjusted with KO H. Filter sterilize.

C-21: Citric acid, 50 mg in 50 ml H_2O, and tri-potassium citrate, 250 mg, pH 5.0. Added to medium and final pH brought 5.5 with KO H. Filter sterilize.

Abbreviations used for Media:

B_5: Medium described by GAMBORG *et al.* (1968).

B_{II}: Medium described by NORSTOG (1973).

R-M-IS: Medium used by ISLAM and SPARROW (1974) based on Randolph's medium described in MORRISON *et al.* (1959).

C-17: Medium used by Jensen based partially on B^{II} and other media used as liquid culture on small and non-uniform monoploid embryos.

C-21: Medium used by JENSEN (1974b) on uniform, well developed embryos.

Appendix 2. Hoagland solution, modified for barley shoot culture

Chemical	Stock solution gm/l	Final solution use ml/l
KH_2PO_4	38.0	1 ml/l
$MgSO_4 \cdot 7H_2O$	52.0	
KNO_3	66.0	
$Ca(NO_3)_2 4H_2O$	94.0	
H_3BO_3	2.86	1 ml/l
$ZnSO_4 \cdot 7H_2O$	0.22	
$CuSO_4 \cdot 5H_2O$	0.10	
$Na_2MoO_4 \cdot 2H_2O$	0.05	
Fe EDTA	12.0	1 ml/l

Buffer: 2-morpholino ethanesulfonic acid MES 19.5 g + 2.0 g NaOH, in one 1 H_2O. Adjust to pH 6.5 with NaOH. Use 5 ml/l nutrient solution. For original Hoagland solution see HEWITT, 1966.

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Chapter III

Cytology, Cytogenetics and Plant Breeding

1. Cytogenetics of Differentiation in Tissue and Cell Cultures

F. D'AMATO

1. Introduction

Working independently of each other, WHITE (1939), GAUTHERET (1939), and NOBÉCOURT (1939), almost simultaneously announced successful methods for the unlimited in vitro growth of plant tissues. Since this initial discovery, the most varied types of tissues and cells from an extraordinary number of plant species have been grown in vitro using solid and/or liquid culture media. In seed plants, in vitro cultures have been obtained from such diverse materials as: ovaries, ovules, and nucelli; excised embryos; seeds; differentiated portions of roots, stems, rhizomes, tubers, bulbils, leaves, flowers, and inflorescences; tumor tissues; apical meristems; pith parenchyma; secondary phloem; pericarp and endocarp of fruits; endosperm; female gametophytes of Gymnosperms; anthers; pollen grains; wall deprived cells (protoplasts) (see D'AMATO, 1972a; MURASHIGE, 1974).

With increasing knowledge on the factors conditioning organogenesis in vitro and improvements in technique, plant propagation through tissue and cell cultures has been achieved in a wide range of species of seed plants, including many economically important crops (review in MURASHIGE, 1974). In this article, the nuclear cytology of tissue and cell cultures and of the plants regenerated therefrom will be discussed. For previous reviews on the nuclear cytology of plant tissue and cell cultures, reference is made to PARTANEN (1963), D'AMATO (1965, 1975), SUNDERLAND (1973a) and SHERIDAN (1975).

2. Plant Regeneration from Shoot Apex Cultures

An essential characteristic of vascular plants is unlimited growth, which is made possible by the localization of apical meristems at the tip of axial organs (shoots and roots) that occurs during embryogenesis. Unlimited growth makes separation of soma cells and germ cells in plants impossible: in seed plants, apical meristem cells give rise to the sporogenous tissues of the flower (germ line), at the time of transition of the shoot apex from the vegetative to the reproductive phase. In a developmental system of this sort, genetic stability of the germ line equals genetic stability of the meristematic cell line. This is ensured by two major properties of meristematic cells: (1) the strict control of the sequence DNA synthesis-mitosis which does not allow extra duplications of DNA that are responsible for somatic polyploidy, and (2) the continuous divisions which eliminate at least part of the spontaneously occurring chromosome structural changes and other genetic defects impairing the reproductive ability of cells (for a further elucidation of the above discussion, see D'AMATO, 1975).

The genetic stability of the meristem line can be, and indeed is, exploited for the maintenance of genetic stocks by means of in vitro culture methods. To MOREL (1960, 1964, 1965) goes the credit of having inaugurated the technique of plant propagation through shoot apex culture. In his experiments on the recovery of virus-free orchids from infected plants, MOREL observed that the shoot meristem (apical dome and a few leaf primordia) of *Cymbidium* and other orchid species, when grown in vitro, developed into one or several protocorms ("regeneration protocorms") very similar to the protocorms of germinating orchid seed ("germination protocorm"). These protocorms were multiplied substantially after a number of recultures. Further development of the protocorms resulted in the production of numerous clonal plants in a short time. MOREL'S technique, sometimes strongly modified, has been extended to many other orchid genera (see Chap. I. 2, 3 of this Vol.).

Progress in the in vitro propagation through shoot apex culture of plants other than orchids has been rather slow; but recently many plant species have been propagated with this method (see MURASHIGE, 1974). In general, plant clones derived from shoot apex cultures are phenotypically homogeneous, thus indicating genetic stability. Since, however, for some characters at least, no phenotypic change is to be expected at the polyploid level, a more satisfactory means of ascertaining nuclear stability in a clone is chromosome number determination. In the few materials in which chromosomes were counted—*Asparagus officinalis* (MURASHIGE *et al.*, 1973), *Gerbera jamesonii* (MURASHIGE *et al.*, 1974), and *Hordeum vulgare* (CHENG and SMITH, 1975)—all regenerated plants were found to be diploid. The experiments on *A. officinalis* clearly demonstrate the importance of plant propagation through shoot apex culture when genetic stability is required: a high frequency of asparagus plants previously regenerated from callus and suspension cultures was found to deviate from the original plants, most of them being tetraploid (WILMAR and HELLENDOORN, 1968; MALNASSY and ELLISON, 1970; Table 1). In cases of genetic variability among plants of clones produced via shoot apex culture—e.g. some of the *Chrysanthemum morifolium* material used by EARLE and LANGHANS (1974)—in addition to a cytological analysis, the possibility should be explored that the explanted shoot apex is a periclinal chimera. That shoot apex cultures, when growing as a callus, can lead to uncovering of chimeras has been documented for the carnation periclinal chimera "White Sim" (HACKETT and ANDERSON, 1967).

Besides ensuring genetic stability, the shoot apex culture allows the establishment of pathogen-free clones; the technique is, therefore, expected to be of great help in the long-term conservation of genetic stocks in gene banks of vegetatively propagated plants (D'AMATO, 1975; MOREL, 1975).

3. Nuclear Cytology of Tissue and Cell Cultures

When a plant tissue, a portion of an organ, or an organ is explanted in vitro, the nuclear conditions found in the culture partly reflect the nuclear condition in vivo (that is, in the primary explant) and partly result from nuclear changes which may occur at the time of callus induction and/or during further growth in vitro.

A very extensive literature is now available on nuclear conditions in differentiated plant tissues and cells, as ascertained by microscopic analysis of experimentally induced mitosis in vivo and, in some materials, by cytophotometric measurement of nuclear DNA contents (reviews by D'AMATO, 1952, 1965; GEITLER, 1953; TSCHERMAK-WOESS, 1956, 1971). Of the hundreds of plant species so far investigated, (less than 10%) are characterized by histological differentiations in a diploid condition. In all, or almost all cells, the nuclei are left in the condition in which they were at the end of the mitosis immediately preceding cell differentiation; that is, with the 2C DNA content which is typical of the pre-DNA synthesis phase (G_1) of the diploid cell cycle. Histological differentiation in a diploid condition is encountered more frequently in some families, e.g. *Asteraceae*, than in others: examples are *Lactuca sativa*, *Helianthus tuberosus*, *Helianthus annuus* and *Crepis capillaris*. Recent investigations on the control of the cell cycle in higher plants, reviewed by D'AMATO (1972b) and VAN'T HOF (1974), support the view that proliferating or potentially proliferative cells in a species are genetically determined to arrest in a certain cycle phase; e.g. G_1 in the case under discussion. It seems plausible that, in a given species, the cell cycle control which operates in vivo also operates in vitro. Leaf callus of *C. capillaris*, when analyzed cytologically and cytophotometrically, was found to consist of diploid cells only up to one year of culture (REINERT and KÜSTER, 1966); but, in the course of time, polyploidy began to appear and increased with time to reach 28% after 20 months (SACRISTÁN, 1971). Since *C. capillaris* does not seem to undergo extra duplication(s) of DNA (endoreduplication) in its tissues in vivo, polyploidization in vitro in this species most probably results from mechanisms other than endoreduplication, such as restitution nucleus formation and/or spindle fusion in binucleate or multinucleate cells (see below). Another plant species studied recently, *Lilium longiflorum*, shows stability at the diploid level in long-term culture (SHERIDAN, 1974, 1975). In both *Crepis* and *Lilium*, however, stability at or around the diploid level does not exclude changes in karyotype due to chromosome aberrations (SACRISTÁN, 1971; SHERIDAN, 1974, 1975).

In the majority of plant species, differentiated tissues in vivo contain endoreduplicated (endopolyploid) nuclei, that is, nuclei whose chromosomes at interphase have undergone one to several duplications: up to twelve in the polytene chromosome cells of the suspensor of *Phaseolus coccineus* and *Phaseolus vulgaris* (review in NAGL, 1974). The degree of endoreduplication may vary from tissue to tissue and in a given tissue it is not uniform, because a variable proportion of cells do not endoreduplicate (either 2C or 4C DNA content); moreover, meristematic cells in differentiated plant parts (pericycle, procambium, cambium) remain diploid. When an explant is brought in vitro, the first mitoses generally reflect the in vivo condition. Diploid nuclei give mitoses with 2n chromosomes (monochromosomes: 2-chromatid chromosomes) and endoreduplicated nuclei give mitoses with 2n diplochromosomes (4-chromatid chromosomes), 2n quadruplochromosomes (8-chromatid chromosomes) or 2n polychromosomes (higher levels of endoreduplication). Diplochromosome and quadruplochromosome mitoses are commonly found in the first phases of in vitro growth, e.g. in stem pith of *Nicotiana tabacum* (NAYLOR *et al.* 1954; PÄTAU and DAS, 1961), in stem internodes of the haploid *Pelargonium* cultivar "Kleiner Liebling" (BENNICI *et al.*, 1968) and in

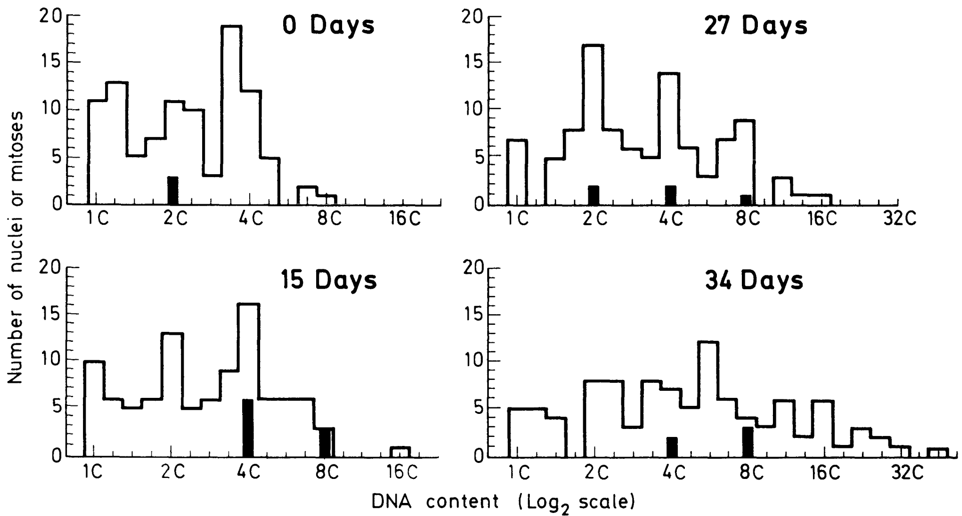

Fig. 1. DNA content in interphase nuclei *(empty bars)* and mitoses *(solid bars)* in the haploid *Pelargonium* cultivar "Kleiner Liebling". Material: internode at explant (zero days) and in callus derived from it after 15, 27 and 34 days. Transfer of the primary explant on fresh medium was made on the 15th day. Note the selection in favor of diploid (*4 C*) and tetraploid (*8 C*) mitoses and the progressive increase in degree of endoreduplication in the interphase nuclei. (From BENNICI *et al.*, 1968)

seedlings of *Haplopappus gracilis* (BENNICI *et al.* 1971). On the contrary, polychromosome mitoses in vitro are only exceptional, because of the great difficulty with which endoreduplicated nuclei of higher levels are induced into mitosis. As in experimentally induced mitoses in vivo, which have been extensively studied (see D'AMATO, 1952), diplochromosome and quadruplochromosome mitoses in vitro give rise respectively to two tetraploid (4x, where x is the basic chromosome number) and two octoploid (8x) nuclei, which by further division produce a tetraploid and an octoploid cell population. By a combined cytological and DNA cytophotometric analysis in the haploid *Pelargonium*, it has been possible to follow in a temporal sequence the nuclear events which occur during development of a callus from the primary explant (Fig. 1).

Since kinetin (K) acts as a trigger for mitosis in endoreduplicated cells (TORREY, 1961; VAN'T HOF and MACMILLAN, 1969) and in cultured pea root cortex cells it can induce endoreduplication prior to mitosis (LIBBENGA and TORREY, 1973), the composition of medium is the most important factor conditioning the composition of the proliferating cell fraction in a culture. Selective induction and maintenance of mitosis in cells of different ploidy levels was first demonstrated in pea root segments. When these were cultured in vitro on Shigemura's synthetic medium, the diploid cells only proliferated; when K (or yeast extract) plus 2,4-dichlorophenoxyacetic acid (2,4-D) were added to the medium, the tetraploid cells were selectively stimulated to mitosis and the proliferating fraction of the culture consisted of tetraploid cells only (TORREY, 1961, 1967; MATTHYSSE and TORREY, 1967). Since a combination of auxin and a cytokinin is essential for DNA

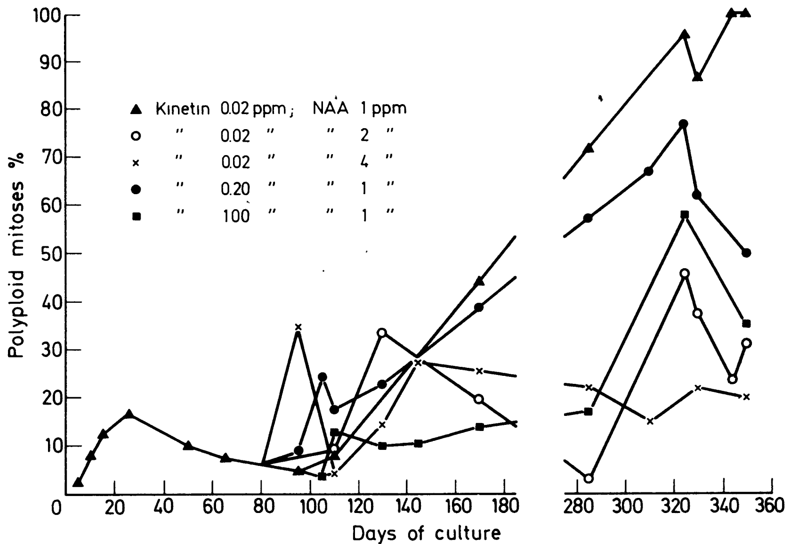

Fig. 2. Frequency of polyploid mitoses at different culture times of calli of *Haplopappus gracilis* grown on Linsmaier and Skoog's medium (1965) containing five different concentrations (in mg/l) of kinetin (K) and naphthalene acetic acid (NAA). The initial callus was grown for 80 days (4 transfers at 20-day intervals) on the medium containing K 0.02 mg/l and NAA 1 mg/l and then subdivided into 5 subcultures. After 350 days in the medium with the lowest K and NAA content, only polyploid mitoses were seen. (From BENNICI *et al.*, 1971)

synthesis and mitosis (SKOOG and MILLER, 1957), their quantitative ratios in a culture medium can greatly influence the composition of the proliferating cell population, as recently shown with *H. gracilis* callus (Fig. 2).

The degree and extent of polyploidy in an in vitro culture tends to increase progressively with increasing age of the primary explant or callus or under particular hormonal regimes (MELCHERS and BERGMANN, 1958; MITRA and STEWARD, 1961; BLAKELY and STEWARD, 1964; MURASHIGE and NAKANO, 1967; DEMOISE and PARTANEN, 1969; BENNICI *et al.*, 1971; MEHRA and MEHRA, 1974). For the non-proliferating cell fraction of calli derived from tobacco stem pith and internodes of haploid *Pelargonium*, it has been shown that some cells may undergo one or two additional endoreduplication cycles during culture (PÄTAU *et al.*, 1957; BENNICI *et al.*, 1968; DEVREUX *et al.*, 1971). Whether endoreduplication may be made responsible for increased polyploidy in the proliferating cell fraction of a culture remains to be investigated. An important mechanism of polyploidization in vitro appears to be restitution nucleus formation due to spindle failure and chromosome lagging at anaphase: these two phenomena have been observed to occur in plant tissue and cell cultures (BAYLISS, 1973). Although not described so far for plant systems in vitro, another mechanism of polyploidization might well be spindle fusion in binucleate and multinucleate cells, which are sometimes present in culture (NAYLOR *et al.*, 1954; MITRA *et al.*, 1960; MITRA and STEWARD, 1961). Spindle fusion in binucleate cells is an important mechanism of polyploidization in the mammalian liver (NADAL and ZAJDELA, 1966; WHEATLEY, 1972); in

plants, this polyploidization mechanism is well documented for the binucleate cells induced by caffeine in root apical meristems (LOPEZ-SAEZ *et al.*, 1965).

Among the various ploidy levels occurring in cultures of somatic plant tissues, of special interest are odd-ploid chromosome numbers: haploid, triploid, pentaploid etc. Haploid and triploid mitoses were reported to occur in in vitro cultures of *H. gracilis* (MITRA and STEWARD, 1961; SHAMINA, 1966); a callus strain that showed 13% triploid mitoses ($3x=6$) at the fourth month of culture (SHAMINA, 1966), when examined several years later, was found to comprise a wide range of ploidy from diploidy ($2n=2x=4$) to $9x$ and higher (SIDORENKO and KUNAKH, 1970). In tobacco, three out of four callus strains with a predominance of triploid (72 chromosomes) or near-triploid cells (FOX, 1963; SHAMINA *et al.*, 1966; SACRISTÁN, 1967; SACRISTÁN and MELCHERS, 1969) have been found to be autotrophic for auxin. Although odd-ploid chromosome numbers may result from nuclear fusion (SUNDERLAND, 1973a), a more probable mechanism for their origin is genome segregation during polyploid mitoses. Genome segregation, which emerges as a multipolar mitotic figure, is known to produce odd-ploid chromosome numbers from dividing polyploid cells in the rat liver (GLÄSS, 1957; MARQUARDT and GLÄSS, 1957) and in mammalian cell cultures (see PERA, 1970). Thus, e.g. in tissue cultures of *Microtus agrestis*, tetraploid cells, besides dividing with a normal bipolar spindle to produce two tetraploid daughter nuclei (for brevity, 4:4), may also divide by a tripolar spindle (1:3:4; 3:2:3; 2:4:2) or a tetrapolar spindle (2:2:2:2). In the same material, haploid cells may originate from a diploid nucleus through a tripolar mitosis (1:2:1) (see PERA, 1970). Another mechanism for the origin of haploid from diploid cells is homologous segregation, which was discovered and fully analyzed in root apices by HUSKINS and his associates (HUSKINS, 1948; WILSON and CHENG, 1949; HUSKINS and CHENG, 1950). The process occurs by separation of the diploid chromosome complement into two truly haploid (homologous) chromosome groups, each of which organizes a separate spindle ("twin spindle") in the cell: the result of this twin mitosis are four haploid nuclei in one and the same cell.

Another genomic change which occurs frequently in in vitro cultures is aneuploidy. In general, aneuploidy is not present, or occurs with extremely low frequency, in the primary explant, because mature plant cells are the direct descendants of meristematic cells that are practically immune from aneuploidy. Aneuploidy is, however, to be expected in mature tissues in plants of particular genetic constitution such as hybridity, e.g. *Saccharum* species hybrids (HEINZ *et al.*, 1969), or polyploidy of recent origin, e.g. tetraploid *Ribes nigrum* (VAARAMA, 1949). Extensive aneuploidy may be produced in the first phases of callus induction in a primary explant. A case in point is the *Nicotiana* stem pith (parenchyma only). In stem pith of *N. tabacum* cultured on White's basic medium supplemented with indole acetic acid (IAA), NAYLOR *et al.* (1954) found a continuous increase of "nuclear bodies" per cell beyond 150 h which "was probably not dependent on normal mitoses alone" ... (NAYLOR *et al.*, p. 28); in the same tissue, cultured on a medium supplemented with IAA (2 mg/l) and kinetin (0.5 mg/l), DAS *et al.* (1958) found that, whilst the mitotic index between 2 and 6 days increased from 2 to 3.6 the percentage of "new cells" increased from about 3–4% to about 50%. More recently, NUTI RONCHI *et al.* (1973), working on the stem pith of *N. glauca* cul-

tured on a synthetic medium supplemented with 2,4-D, observed a very active nuclear fragmentation (amitosis) between the second and the sixth day, which resulted in multinucleate cells with differently sized nuclei; meanwhile, only occasional mitoses were present in the explant. If mitoses followed amitosis, a wide range of chromosome numbers should occur: indeed, in tobacco pith cultured Murashige and Skoog's basal medium (1962) containing IAA 2 mg/l and K 0.2 mg/l, the first mitotic wave (which occurred at 6 days) showed chromosome numbers ranging from 40 to 215 with "no clear mode ... in the array of the chromosome number" (SHIMADA and TABATA, 1967). These results are in striking contrast to the experiments of PÄTAU and DAS (1961), in which the first mitotic wave in the pith explant (medium supplemented with IAA 2 mg/l and K 0.5 or 1 mg/l) occured at the second day and the mitoses observed in the first 6 days were all euploid (diploid, tetraploid and octoploid). That the very wide range of aneuploidy observed by SHIMADA and TABATA (1967) does not reflect a preexisting condition in the pith, as claimed by these authors, but originates in the first phases of callus induction, is clearly demonstrated by the DNA cytometric analyses in vivo: the tobacco pith has been shown to consist of cells with "euploid" DNA contents only: 2C, 4C, 8C, and 16C (PÄTAU *et al.*, 1957; DEVREUX *et al.*, 1971). Aneuploidy also originates during in vitro culture, as clearly shown by a progressive increase in frequency and extent of aneuploidy with increasing age of the cultures: e.g. *H.gracilis* (BLAKELY and STEWARD, 1964), carrot (MUIR, 1965), pea (TORREY, 1967), tobacco (MURASHIGE and NAKANO, 1965, 1967). Of particular interest for the problem of chromosome number stability in established cultures is the observation that in some species, e.g. tobacco, wheat, and rice, the degree and extent of aneuploidy depend on the organ from which explants are taken (SHIMADA, 1971; YAMADA *et al.*, 1967). In the establishment and maintenance of certain aneuploid chromosome complements non-random selection of particular chromosomes takes place (SINGH *et al.*, 1972).

Chromosome structural changes of different types (deficiency, translocations, dicentric chromosomes, isochromosomes) are not rare in plant tissue and cell cultures; e.g. in *Lolium perenne* (NORSTOG *et al.*, 1969), *Daucus carota* (MITRA *et al.*, 1960), *H.gracilis* (MITRA and STEWARD, 1961), *Triticum aestivum* and *Triticum monococcum* (KAO *et al.*, 1970), *Crepis capillaris* (SACRISTÁN, 1971), *Lilium longiflorum* (SHERIDAN, 1974, 1975). In these materials, some karyotypes with changed chromosomes apparently have a selective advantage. As shown in a callus strain of *Haplopappus*, persistence in culture of a dicentric chromosome is ensured through the well-known breakage-fusion-bridge cycle (SUNDERLAND, 1973a). Although chromosome structural changes generally originate during in vitro culture, there is evidence that deficiencies and dicentric chromosomes also occur in mature plant cells in vivo (D'AMATO and AVANZI, 1948; LEVAN and LOTFY, 1949).

4. Plant Regeneration from Tissue and Cell Cultures

When plants are regenerated from in vitro cultures, plant regeneration most frequently occurs via formation of adventitious shoots and rarely via embryo formation (MURASHIGE, 1974) (see Table 2). It is not yet known for certain

Table 1. Chromosome number of plants or shoots regenerated from callus or suspension cultures originating from explants of somatic tissues and, when known, chromosome number in the cultures used for plant regeneration. In case of different chromosome numbers, the number more frequently observed is shown. For triploid plants regenerated from endosperm cultures, see Chapter III.4 of this Vol.

Species, chromosome number and type of culture	Chromosome number in culture	Chromosome number of regenerated plants or shoots	Reference
Asparagus officinalis ($2n=20$), Callus, suspension	Not reported	20, *40*	WILMAR and HELLENDOORN (1968) TAKATORI *et al.* (1968), MALNASSY and ELLISON (1970)
Brassica oleracea ($2n=18$), Callus	Not reported	18, 36	HORÁK *et al.* (1971), HORÁK (1972)
Daucus carota ($2n=18$), Suspension	9, 18, 36, 72	18	MITRA *et al.* (1960)
Hederocallis flava ($2n=22$)	Not reported	22	STEWARD *et al.* (1963) CHEN and GOEDEN (1974)
Lilium longiflorum ($2n=24$), Callus	24, 24+1 isochrom., 24 including 1 isochrom.	24, 48, 24+1 isochrom.	SHERIDAN (1974, 1975)
Nicotiana alata ($2n=18$), Callus	Not reported	18, 19	NISHIYAMA and TAIRA (1966)
N. tabacum ($2n=48$),			
Tn_1, auxin heterotrophic callus	47–49, 83–85, 92–100 (mode at 96)	65–71	
Tn_2, auxin heterotrophic callus	47–49, 83–96, 182–184 191–193 (mode at 92)	82–90	SACRISTÁN (1967), SACRISTÁN and MELCHERS (1969)
Ta, habituated callus	35–37, 65–82, 143–145 (mode at 72)	57–65 (*61*)	
Tc, crown gall callus	35–37, 47–53, 68–70, 71–76, 80–82 (mode at 72)	60–70 (*63*)	
S_{17}, habituated callus	Not reported	50–60	SACRISTÁN and LUTZ (1970)
S_{21}, slightly habituated callus	Not reported	94–97 (*96*)	
Pith callus	Not reported	48, *96*, Aneuploid	MURASHIGE and NAKANO (1966)
N. tabacum, haploid ($n=2x=24$), Stem callus	Not reported	24, *48*, 96	DEVREUX *et al.* (1971), KOCHHAR *et al.* (1971) NITSCH (1972)

Table 1. (continued)

Species, chromosome number and type of culture	Chromosome number in culture	Chromosome number of regenerated plants or shoots	Reference
Callus from young leaves	Not reported	24, 48	KASPERBAUER and COLLINS (1972)
Callus from aged leaves	Not reported	48	
Oryza sativa ($2n=24$)			
Callus from seed	Wide variation	24	NISHI *et al.* (1968)
Callus from ovary	Not reported	48	NISHI and MITSUOKA (1969)
Pelargonium zonale, haploid ($n=x=9$), Callus	9, 18, 36	9, *18*	BENNICI (1974)
Populus tremula ($2n=4x=76$), Callus	Not reported	76	WINTON (1971)
P. tremuloides ($2n=3x=57$), Callus	Not reported	57	WINTON (1970)
Prunus amygdalus ($2n=16$), Callus	9, 15, 16, 24, 28, 32	16	MEHRA and MEHRA (1974)
Saccharum species hybrids			
H37–1933 ($2n=106$), Callus	Not reported	106	HEINZ *et al.* (1969), HEINZ and MEE (1971)
H50–7209 ($2n=108$–128)[a], Callus	71–90, 101–110, 154–160, more than 300	*94–120*[a], 17–118[a]	
Triticum aestivum ($2n=42$), Callus	26–84 (mode at 42)	42	SHIMADA *et al.* (1969)

[a] Chromosome mosaicism in one and the same plant.

whether the nuclear conditions in an in vitro culture may be a determinant of the regenerative (organogenic or embryogenic) potential of the culture. In his study of long-term callus cultures of *Pisum sativum*, TORREY (1967) observed a progressive loss in organogenic capacity with increasing polyploidy and aneuploidy; in tobacco callus, the capacity for shoot formation was found to be severely reduced in callus strains with an extensive aneuploidy (MURASHIGE and NAKANO, 1967; ZAGORSKA *et al.*, 1974). The suggestion of TORREY (1967) and MURASHIGE and NAKANO (1967) that loss in organ-forming capacity is correlated with increasing variation in chromosome number does not find confirmation in the experiments of SACRISTÁN and MELCHERS (1969) and SACRISTÁN and LUTZ (1970); these demonstrate a very easy regeneration in vitro of a wide variety of aneuploid tobacco plants (Table 1). Obviously, the control of morphogenetic ability in plant tissue and cell cultures is not wholly genetic, but depends at least partially on epigenetic changes which occur during growth and differentiation in vitro (MEYER-TEUTER and REINERT, 1973).

Information on the nuclear cytology of plants regenerated in vitro from somatic tissue and cell cultures is limited to a dozen species; for a few of these, the nuclear conditions of the cultures used in regeneration experiments are known (Table 1). Despite the occurrence in culture of different ploidy levels and/or aneuploidy, only diploid plants were regenerated in *D. carota*, *Oryza sativa*, *Prunus amygdalus*, *T. aestivum*. In *L. longiflorum*, which keeps relatively high stability at the diploid level in long-term cultures, 1 out of 8 regenerated plants was tetraploid, 5 were diploid ($2n=24$) with normal karyotype and 2 had $24+1$ isochromosome; in any case, the karyotype of the regenerated plants was identical to the karyotype of the original callus. In other species, also polyploid and/or aneuploid plants have been regenerated in vitro; in some materials, e.g. *N. tabacum* and *O. sativa*, the ploidy level of the regenerated plants seems to be dependent on the type of primary explant used. A wide range of aneuploidy has been found among in vitro regenerated plants of the *Saccharum* species hybrid H 50–7209 and in *N. tabacum*. All plants regenerated in vitro from the *Saccharum* hybrid were chromosomal mosaics; a condition which reflected the chromosomal mosaicism of the hybrid in vivo. In *Nicotiana*, a wide variety of callus types were used for plant regeneration in vitro; an interesting phenomenon, which is unexplained, is that the chromosome number of plants regenerated from calluses with a wide aneuploidy range is in all cases lower than the chromosome number most frequent in the culture (mode number), (SACRISTÁN and MELCHERS, 1969).

In general, the origin of in vitro regenerated plants traces back to a single cell. In carrot, the development of a somatic cell into an embryo has been followed by microcinematography (BACKS-HÜSEMANN and REINERT, 1970). For plants regenerated through adventitious shoot formation, a single-cell origin has been inferred from the occurrence of the same chromosome number in the root and shoot meristem (or leaf primordia) of each plant (SACRISTÁN and MELCHERS, 1969; MALNASSY and ELLISON, 1970), or from an analysis of microsporocytes in different flower twigs in the plant (BUTENKO *et al.*, 1967). Diplotetraploid mosaicism has been observed in 2 out of 17 in vitro regenerated plants of *Brassica oleracea* (HORÁK, 1972), whereas an extensive chromosomal mosaicism (for four or five chromosome numbers) has been observed in a few of the hundreds tobacco

plants regenerated in the experiments of SACRISTÁN and MELCHERS (1969). In the absence of appropriate analyses on the size of sectors with different chromosome numbers in the mature plant, chromosomal mosaicism cannot be taken as evidence for a pluricellular origin of the regenerate. The possibility that some changes in chromosome number occur during in vitro growth of the regenerate is strongly suggested by the observations of BENNICI (1974) on haploid *Pelargonium.*

5. Plant Regeneration from Anther and Pollen Cultures

Since the initial discovery of GUHA and MAHESHWARI (1964, 1966) on *Datura innoxia*, conditions have been established for the induction of growth in pollen of many species of Angiosperms (see SUNDERLAND, 1973b; Chap. II.1).

Table 2 summarizes the available information on the ploidy status of the plants regenerated from anther and pollen cultures and their mode of development, either through direct embryo formation from a pollen grain or through adventitious shoot formation from a callus which generally develops from pollen. For some of the cases of induction of plants with different ploidy levels from a callus, it has been suggested that the callus was partly of anther and partly of pollen origin. But, in some cases at least—e.g. rice (NIIZEKI and OONO, 1971), barley (CLAPHAM, 1973), and *D.metel* (NARAYANASWAMY and CHANDY, 1971)—the pollen origin of the callus is well documented. Non-haploid (diploid, triploid, tetraploid, pentaploid, hexaploid) embryos and plants have been derived from anther culture (Table 2); they may be the result of fusion of vegetative and generative nuclei in pollen grains or of development of unreduced and other types of microspores formed by meiotic irregularities. A spindle fusion mechanism certainly operates in the very frequent production of triploid plants in *Petunia hybrida* and *D.innoxia:* the endoreduplicated generative nucleus (*n* diplochromosomes) and the vegetative nucleus (*n* chromosomes) divide on a common spindle producing two triploid daughter nuclei. Failure of the spindle mechanism or chromosome doubling in a triploid nucleus can explain the formation of a hexaploid plant in *D. innoxia* (SUNDERLAND *et al.*, 1974) and of triploid-hexaploid mosaics in *P.hybrida* (RAQUIN and PILET, 1972). An important point to be stressed is that the diploid and polyploid plants which originate directly from pollen grains are completely homozygous.

In view of the importance of haploids for biochemical genetics and mutagenesis, attention must be paid to methods allowing the long-term maintenance in vitro of haploid callus or the in vitro propagation of haploid plants without loss of haploidy.

6. Plant Regeneration from Protoplasts

Naked plant cells, also called protoplasts, are obtained from cells from which the wall has been removed by physical or, more generally, enzymatic means. Methods for the isolation and in vitro culture of protoplasts have been developed in many

Table 2. Chromosome number of plants regenerated from anther and pollen cultures. In case of differer chromosome numbers, the chromosome number more frequently observed is shown

Species and chromosome number	Mode of development (rare)	Chromosome number of regenerated plants	Reference
Aegilops caudata × *Ae. umbellulata* Amph. ($2n=28$)	Callus	14	Kimata and Sakamoto (1972)
Arabidopsis thaliana ($2n=10$)	Callus	5	Gresshoff and Doy (1972a)
Asparagus officinalis ($2n=20$)	Callus	10	Pelletier *et al.* (1972), Raquin (1973)
Atropa belladonna ($2n=72$)	Embryo	*36*, 72, 108	Zenkteler (1971), Kohlenbach and Geier (1972), Narayanaswamy and George (1972)
Brassica oleracea ($2n=18$)	Callus	9	Kameya and Hinata (1970)
Capsicum annuum ($2n=24$)	Embryo (Callus)	12	George and Narayanaswamy (1973), Wang, Y. Y. *et al.* (1973)
Datura innoxia ($2n=24$)	Embryo	12, *24*, 36, 48, 72	Guha and Maheshwari (1964, 1966), Engvild *et al.* (1972), Nitsch (1972), Sunderland *et al.* (1974)
D. metel ($2n=24$)	Embryo	12, *24*, 36	Narayanaswamy and Chandy (1971), Iyer and Raina (1972), Nitsch (1972)
D. metel, haploid ($n=x=12$)	Embryo	12, *24*	Chandy and Narayanaswamy (1971)
D. meteloides ($2n=24$)	Embryo	*12*, 24	Kohlenbach and Geier (1972), Nitsch (1972)
D. muricata ($2n=24$)	Embryo	12	Nitsch (1972)
Fragaria virginiana ($2n=56$)	Callus	56	Rosati *et al.* (1975)
Hordeum vulgare ($2n=14$)	Callus	7, *14*, 28	Clapham (1971, 1973), Malepsz' and Grunewald (1974)
Lilium longiflorum ($2n=24$)	Callus	12	Sharp *et al.* (1971)
Lolium multiflorum (4*x*)*x* *Festuca arundinacea* (12*x*) ($2n=49$)	Callus	25	Nitzsche (1970)
Lotus corniculatus ($2n=24$)	Callus	*24*, 48	Niizeki and Grant (1971)
Lycium halimifolium ($2n=24$)	Embryo	12	Zenkteler (1972)
Lycopersicum esculentum ($2n=24$)	Callus	12	Gresshoff and Doy (1972b)
Nicotiana alata ($2n=18$)	Embryo	9	Nitsch and Nitsch (1969)
Nicotiana attenuata ($2n=24$)	Embryo	12	Collins and Sunderland (1974
N. glauca × *N. langsdorffii* Amph. ($2n=42$)	Embryo	21	Smith (1974) and personal communication
Nicotiana glutinosa ($2n=24$)	Not reported	12	Nakamura and Itagaki (1973)
Nicotiana knightiana ($2n=24$)	Embryo	12	Collins and Sunderland (1974
Nicotiana otophora ($2n=24$)	Not reported	*12*, 24	Collins *et al.* (1972), Collins anc Sadasivaiah (1972), Nakamur/ and Itagaki (1973)
Nicotiana paniculata ($2n=24$)	Not reported	12	Nakamura and Itagaki (1973)
Nicotiana raimondii ($2n=24$)	Embryo	12	Collins and Sunderland (1974
Nicotiana rustica ($2n=48$)	Embryo	24	Nitsch (1972), Nitsch and Nitsch (1969), Nakamura and Itagak (1973)

Table 2. (continued)

Species and chromosome number	Mode of development (rare)	Chromosome number of regenerated plants	Reference
Nicotiana suaveolens (2*n* = 32)	Embryo	16	SMITH (1974) and personal communication
Nicotiana sylvestris (2*n* = 24)	Embryo	*12*, 24	BOURGIN and NITSCH (1967), NITSCH *et al.* (1968), NITSCH and NITSCH (1969), NITSCH (1972)
N. tabacum (2*n* = 48)	Embryo (Callus)	*24*, 48	BOURGIN and NITSCH (1967), NAKATA and TANAKA (1968), NITSCH *et al.* (1968), NITSCH and NITSCH (1969), TANAKA and NAKATA (1969), BURK (1970), CARLSON (1970), DEVREUX (1970), MELCHERS and LABIB (1970), NILSSON-TILLGREN and WETTSTEIN-KNOWLES (1970), SHARP *et al.* (1971), BURK *et al.* (1972), COLLINS *et al.* (1972), COLLINS and SADASIVAIAH (1972), NITSCH (1972), OPATRNY (1973), KASPERBAUER and COLLINS (1974)
Oryza sativa (2*n* = 24)	Callus	12, *24*, 36, 48, 60	NIIZEKI and OONO (1968, 1971), NISHI and MITSUOKA (1969), GUHA *et al.* (1970), IYER and RAINA (1972)
Pelargonium hortorum (2*n* = 18)	Callus	36	ABO EL-NIL and HILDEBRANDT (1971)
Petunia axillaris (2*n* = 14)	Embryo	*21*, 28	ENGVILD (1973)
Petunia hybrida (2*n* = 14)	Embryo (Callus)	7, 14, *21*, 28	RAQUIN and PILET (1972), WAGNER and HESS (1974)
Secale cereale (2*n* = 14)	Callus	*14*, higher ploidy	WENZEL and THOMAS (1974)
Setaria italica (2*n* = 18)	Callus	9, 18	BAN *et al.* (1971)
Solanum dulcamara (2*n* = 24)	Embryo	12	ZENKTELER (1973)
Solanum melongena (2*n* = 24)	Callus	24	RAINA and IYER (1973)
Solanum nigrum (2*n* = 72)	Callus	*36*, 72, 108, Aneuploid	HARN (1972)
Solanum tuberosum (2*n* = 48)	Embryo	24, 48	DUNWELL and SUNDERLAND (1973), SUNDERLAND, personal communication
Solanum verrucosum (2*n* = 24)	Callus	12	IRIKURA and SAKAGUCHI (1972)
Triticum aestivum (2*n* = 42)	Callus (Embryo)	*21*, 42	OUYANG *et al.* (1973), PICARD and DE BUYSER (1973), WANG C. C. *et al.* (1973)
T. aestivum x *Secale cereale* Amph. (2*n* = 56)	Callus	28	WANG, Y. Y. *et al.* (1973)

plant species (see COCKING, 1972 and Chap. IV.1 of this Vol.). Under appropriate cultural conditions, plant protoplasts can regenerate their wall and divide to form a callus from which plants may be regenerated. In a very extensive experiment on plant regeneration from protoplasts of leaf mesophyll of tobacco ($2n=48$), which was begun by TAKEBE *et al.* (1971), MELCHERS (1974) isolated 2352 diploids, 124 tetraploids ($2n=96$) and an unspecified number of aneuploids. Since deviation from regular chromosome number of 48 is very unusual in tobacco mesophyll cells, it appears that most, if not all, of the deviations from diploidy (tetraploidy, aneuploidy) arose in vitro. Obviously, plant protoplasts in culture are subject to the same nuclear changes as plant cells in callus or suspension culture.

Fusion of protoplasts isolated from two different species, if followed by plant regeneration, is expected to produce a somatic hybrid of amphidiploid nature. Indeed, CARLSON *et al.* (1972) have succeeded in producing in vitro a 42-chromosome somatic hybrid with biochemical and morphological characteristics identical to those of the sexual amphidiploid *N. glauca* × *N. langsdorffii* ($2n=42$). More recently, plants have been regenerated from intraspecific cell hybrids. In tobacco, MELCHERS and LABIB (1974) have succeeded in regenerating 48-chromosome plants from fused protoplasts of two haploid ($n=2x=24$) chlorophyll deficient and light sensitive strains bearing genes, which complement to normal leaf color and resistance to high light intensity both in the sexual and the somatic hybrid. Of the twenty one somatic hybrids studied, ten had 48 chromosomes, seven had a chromosome number in the neighborhood of 70, one was tetraploid, one was triploid and the remaining two had 46 and 90 chromosomes respectively.

The use of genetic complementation for selecting somatic cell (plant) hybrids is a method of great potential value. It remains to be investigated whether genetic complementation may occur also at the interspecific level.

7. Concluding Remarks

From the above discussion it appears that in higher plants the only cell line endowed with genetic stability is the meristematic cell line. Plant propagation by means of the true shoot apex culture (apical dome alone or with a few leaf primordia) is the only procedure which ensures genetic stability in the in vitro propagated progeny. Although such a progeny is expected to be genetically uniform (clone), cytological and genetic analyses on the clones derived from meristem cultures would be of great value in evaluating the in vitro genetic stability of apical meristems; such a knowledge is essential in view of the importance of the shoot apex culture in the long-term maintenance of genetic stocks of vegetatively propagated plants, both in every day practice and in gene banks. A question which should be answered in the future is whether non-dividing apical meristems—either dormant or brought into a stationary phase, in G_1 and/or G_2, by procedures such as those so successfully applied by VAN'T HOF (1974) to apical meristems of excised roots—may be stored for long periods without loss of viability and genetic stability as well. To this end, preservation methods such as submersion under mineral oil and deep freezing (see Chap. VII. 3 of this Vol.) should be tried.

In contrast with the situation in shoot apex cultures, variations in ploidy, chromosome number and karyotype are of common occurrence in plant tissue and cell cultures either of somatic (non-meristem) or germinal (pollen grain) origin. The easy production of doubled haploid and polyploid plants (generally, non-chimerical) from in vitro culture of many plant species leads to recommend (as already suggested by MURASHIGE and NAKANO, 1966) the in vitro technique instead of other methods of chromosome doubling (e.g. colchicine treatment). Since these methods are applied to multicellular structures (e.g. seeds, shoot apices), cytochimeras are obtained from which the isolation of non-chimerical plants may be difficult or time-consuming. It must, however, be stressed that an exploitation of the variability in ploidy levels, and/or in aneuploid chromosome numbers, finds serious limitations in the selective advantage of particular chromosome complements (generally diploid) in the process of plant regeneration in vitro (Table 1). The very sporadic recovery of aneuploids among plants regenerated from tissue cultures of truly diploid species (those in which $2n = 2x$), strikingly contrasts with the very easy regeneration of aneuploids from tissue cultures of polyploid species (e.g. *N. tabacum*) or *Saccharum* species hybrids (Table 1). Selective advantage in regeneration of the diploid chromosome number raises the question whether the karyotype of the original material is preserved. Unfortunately, knowledge on this very important aspect of genetic stability (cf. D'AMATO, 1975) is so far limited to only one species, *Lilium longiflorum* (SHERIDAN, 1974, 1975).

References see page 442.

2. DNA Amplification and Tissue Cultures

M. BUIATTI

1. Introduction

According to several authors (AMALDI and LAVA-SANCHEZ, 1975) DNA sequences in Eukaryotes can be resolved into three distinct classes differing in the degree of redundancy: highly repetitive, intermediate, and unique. Highly repetitive sequences are not transcribed and seem to be devoted to some as yet unknown regulatory function, whereas to the third class seem to belong structural genes *sensu strictu*. The intermediate group, on the other hand, comprises interspersed short sequences and "reiterated" clusters of genes. Several hypotheses have been put forward as to the function of the first kind of sequences (GUILLE and QUETIER, 1973; WALLACE and KASS, 1974), most of them implying some kind of control function or regulatory role on transcription and translation processes.

Gene clusters have also been found mostly for "housekeeping" genes (as reviewed by AMALDI and LAVA-SANCHEZ, 1975) as those for ribosomal RNA, 5s RNA, t-RNA, histone messenger RNA, the multiplicity of which seems to be under genetic control. Up to now, however, no unifying theory has been proposed which might explain in teleological terms the reason for the large differences in multiplicity at the inter- and intraspecific levels observed for the forementioned genes in several plant species, and their influence (if any) on plant developmental processes. In this context plant tissue cultures, with their great potential as a dynamic developmental test system, have been exploited only very recently, although results are already available which seem to shed a new light on the problem of the function of amplification and its relation to redundancy (reiteration). The difference between these two phenomena seems to consist mainly in the fact that the first is considered as a transient process directly connected with specific developmental stages, whereas under the second headline are generally listed all the differences in multiplicity at the inter- and intraspecific level which are heritable. The scope of this review is to look at this problem in a dynamic way, utilizing all the existing evidence in an attempt to answer the question of the existence of a unified developmental role of information redundancy. This is certainly not beyond the scope of this book, as plant tissue cultures offer a material particularly suited for the study of this problem, mainly because of their ontogenetic plasticity in response to external treatments. In this sense they allow parallel observations on the effect of hormones, or of other controlled variations in the environment, on differentiation and dedifferentiation on the one hand, and on amplification processes on the other.

Moreover, the fact that plant tissue cultures can be grown on synthetic media under strictly controlled conditions opens the possibility of directly and differentially influencing the amplification phenomenon itself. For this purpose the re-

view will be divided into three subheadings, the first being concerned with transient amplification phenomena, the second with the data showing the induction during development of unstable heritable redundancy (semipermanent reiteration), the third with fully heritable reiteration differences.

2. Transient Amplification

By transient amplification we shall mean all those phenomena which directly or indirectly suggest the selective replication of specific DNA portions concomitant with specific changes in developmental patterns and limited in time. More broadly, under this heading we shall collect the existing evidence on DNA synthesis not connected with mitotic processes or with endopolyploidization, and apparently needed for the expression of morphogenetic phase changes. Table 1 summarizes the data so far available on these processes, a part of which, however, is only of indirect relevance. For the sake of simplicity the existing evidence is divided into (1) amplification during differentiation and in specialized cells, and (2) extra-DNA synthesis in materials undergoing dedifferentiation and cell proliferation.

Table 1. Direct and indirect indications of transient DNA amplification in plants

Material	Evidence	Reference
Helianthus tuber slices	Inhibition of expansion growth by DNA synthesis inhibitors	KAMISAKA and MASUDA (1970)
Sinapsis alba seedling hypocotyl segments	Inhibition of elongation by 5-FUDR	CAPESIUS *et al.* (1972)
Kalanchoe daigremontiana seedlings	Inhibition of elongation by 5-FUDR	BOPP (1970a, b)
Lens caulinaris seedlings	Inhibition of GA induced elongation by 5-FUDR	NITSAN and LANG (1966)
Cucumis sativus seedling	Inhibition of auxin and GA induced elongation by 5-FUDR	DEGANI *et al.* (1970) DEGANI and ATSMON (1970)
Nicotiana tabacum pith sections	DNA synthesis requirement for auxin induction of cell enlargement	MAHESHWARI and NOODEN (1971)
Vicia faba primary root	Loss of labeled DNA during differentiation	MACLEOD (1973)
Spinach stems	Loss of labeled DNA in aging stems	ANKER *et al.* (1971)
Lycopersicon esculentum collenchyma tissue	DNA turnover	HURST *et al.* (1971)
Hordeum vulgare roots	Aluminium induced metabolic DNA synthesis	SAMPSON *et al.* (1965)

Table 1. (continued)

Material	Evidence	Reference
Allium cepa roots	rDNA amplification associated with metaxylem differentiation	INNOCENTI and AVANZI (1971); AVANZI *et et al.* (1973)
Lilium, Trillum, Paeonia, Tradescantia, Gasteria, Rhoeo, Hosta, Allium, microsporocytes	Feulgen positive bodies extrusion into the cytoplasm	SPARROW and HAMMOND (1947)
Cucumis melo, Cucumis sativus root and fruits	Different percentages of satellite	PEARSON *et al.* (1974)
Germinating wheat embryos	Loss of ribosomal genes during germination	CHEN and OSBORNE (1970)
Wounded *Vicia faba* stems	DNA synthesis without cell division after wounding	KUPILA and THERMAN (1971)
Lens, Cucumis, Lycopersicon, Glycine max elongation roots and stems	Synthesis of a DNA satellite	QUETIER (1970)
Cucurbita melo seeds	Synthesis of a DNA satellite during cold stress	QUETIER (1970)
Wounded stems from 12 dicotyledonous species	Synthesis of a DNA satellite after wounding	GUILLÉ (1972)
Phaseolus coccineus roots, shoots, integuments, suspensors	Different percentages of satellite DNA in different tissues	LIMADE FARIDA *et al.* (1975)
Phaseolus suspensor	Cytological and biochemical evidence of amplification	NAGL (1974); AVANZI *et al.* (1970); AVANZI *et al.* (1972)
Calystegia soldanella primary root quiescent center	Cytological evidence of localized DNA synthesis	AVANZI *et al.* (1974)
Solanum tuberosum tuber tissue in vitro	DNA synthesis without mitosis	WATANABE and IMASEKI (1973)
Lactuca sativa cotyledons in vitro	Cytological evidence of nucleolar extrusion and localized DNA synthesis	NUTI RONCHI and GREGORINI (1970)
Helianthus tuberosus tuber tissue in vitro	Early DNA synthesis	YASUDA *et al.* (1974)
Cymbidium maturing parenchyma in vitro	Cytological and biochemical evidence of DNA amplification and extrusion into the cytoplasm	NAGL (1972), personal communication
Nicotiana langsdorffii, N. bigelovii pith tissue in vitro	Cytological evidence of nucleolar extrusion	NUTI RONCHI and MARTINI, unpublished
N. glauca and *N. glauca* × *N. langsdorffii* (non tumorous) pith tissue in vitro	Cytological and biochemical evidence of DNA amplification and extrusion into the cytoplasm	NUTI RONCHI *et al.* (1973); PARENTI *et al.* (1973)
Ginkgo biloba female gametophyte	Cytological evidence of localized DNA synthesis	AVANZI and CIONINI (1971)
Marsilea root apices and primordia	Cytological evidence of localized DNA synthesis	AVANZI and D'AMATO (1970); SOSSOUNTZOV (1969)

2.1 Amplification in Differentiating and Differentiated Systems

A series of experiments giving merely indirect proof of DNA amplification, mainly based on the need of non-mitotic DNA synthesis for specific growth and differentiation patterns, e.g. cell elongation and expansion, are described here.

NITSAN and LANG (1966) were the first to stress the importance of DNA synthesis for gibberellic-acid-induced elongation of Lentil epicotyl, on the grounds of FUDR inhibition of the process. These results were questioned by HOLM and KEY (1969), but confirmed in a study on cucumber hypocotyl elongation by DEGANI and ATSMON (1970), and DEGANI *et al.* (1970). Contemporarily BOPP (1970a, b) carried out a series of experiments on the elongation of *Sinapis alba* and *Kalanchoe daigremontiana*, which showed the need of an early DNA replication preferentially localized in chromocenters.

Further work on *Sinapis*, moreover, limited to few hours—the period sensitive to FUDR, thus supporting the view of the existence of a fairly specific moment in the DNA synthetic pattern (CAPESIUS *et al.*, 1972). All the studies on gibberellic acid or auxin-induced expansion without cell division, so far reported do not allow speculation on the nature of the synthesized DNA. The only exception to this rule can be found in the work of QUETIER *et al.* (1968) who observed the appearance of a G+C rich DNA satellite at least partially coding for ribosomal RNA during root and hypocotyl elongation of *Lens caulinaris*.

Another cell differentiation process where an important role for gene amplification has been suggested is xylem differentiation. Studying this process, from some data which indicated an ill-defined DNA synthetic requirement in the first phases of xylogenesis, INNOCENTI and AVANZI (1971) observed in *Allium cepa* localized ^{3}H-thymidine labeling around the nucleolus followed by nucleolar extrusion into the cytoplasm and chromatin accumulation around the nucleoli. Furthermore, cytological hybridization experiments carried out with a mixture of 18S and 25S ^{3}H-rRNA showed the ribosomal nature of the nucleolus-like extruded bodies, suggesting the existence of a sequence of events leading to xylogenesis, one of the necessary steps being an extra replication of DNA sequences coding at least in part for ribosomal r-RNA.

A similar temporal sequence has also been found for another example of cell maturation, the parenchyma of *Cymbidium* protocorms cultured in vitro. Early results by ALVAREZ (1968, 1969) suggested to NAGL and RÜCKER (1972) a thorough analysis of the process of protocorms maturation and of the possible influences on it of hormonal treatments. For this purpose protocorm fragments were cultured under in vitro conditions and observed with current cytological techniques. Nuclei in root hair and parenchyma cell were found to show disproportionately large chromocenters and DNA values exceeding those expected from a normal endopolyploidization process. Moreover, whereas GA_3 (gibberellic acid) stimulated mitotic activity and IAA did not seem to exert any effect at the nuclear level, 2,4-D inhibited mitotic activity but enhanced the amplification. This effect of 2,4-D also resulted in a strong inhibition of morphogenetic processes leading, at the 4.5×10^6M concentration, to the loss of root hair, a striking decrease in the frequency of plantlet formation, and the persistance of large quasi-amorphous tissue masses with few, unorganized vascular elements. Further work,

particularly concerned with parenchyma maturation (NAGL, 1972), showed the existence of two distinct cell populations in this tissue. One of them (the "standard" population in NAGL's terminology) underwent endopolyploidization following a normal geometrical series; the other, exhibited heterochromatin-rich nuclei which showed disproportionately high DNA values. The number of chromocenters in the last population was constant but their DNA content as measured microspectrophotometrically was much higher than in standard nuclei and, furthermore, ^{3}H-thymidine incorporation was localized almost exclusively in the chromocenters. The extra-DNA-containing cells were found to be mainly green and assimilating, whereas the "normal" ones were observed only in storage parenchyma (NAGL *et al.*, 1972). The amplification process thus described was found to be restricted to the first week after the start of new cultures and to be inhibited by a DNA inhibitor like hydroxyurea, but not by Actinomycin D. Light and electron microscopic observations suggested that "amplified" nuclei eventually lose their extra DNA, releasing it into the cytoplasm which, after about 12 h, is labeled slightly throughout (NAGL and CAPESIUS, personal communication). Only preliminary data are available on the nature of the synthesized DNA; they seem to suggest the possible involvement of an A-T rich satellite (1.682 g/cm^3) buoyant density (CAPESIUS *et al.*, 1975).

Besides the reported examples of transient DNA amplification occurring during a dynamic differentiation process, a few well-studied cases can be cited which point to the existence also in plants of extra-DNA synthesis in highly specialized cells. This is so in the case of *Phaseolus coccineus* suspensor, an organ where giant cells with extensive endopolyploidization (polyteny), are known to occur. In this material NAGL (1969, 1970) first showed a specific puffing pattern induced by the temperature and nucleolar extrusion accompanied by the formation of additional nucleoli. In a very detailed cytological study AVANZI *et al.* (1970), besides confirming these data, observed extra DNA synthesis not only in the nucleolus organizing regions but also in other SAT and non-SAT chromosomes. Further analyses, based on cytological r-RNA-DNA hybridization (AVANZI *et al.*, 1972), revealed that only micronucleoli produced by the chromosome pair S_1contained rDNA, the S_2 pair DNA bodies being apparently devoid of such sequences. To obtain more data on the nature of the amplified DNA, analytical ultracentrifugation was carried out by LIMA-de-FARIA *et al.* (1975) and this suggested the appearance of a specific DNA satellite at 1.696 g/ml not observed in other tissues of the same plant, and representing about 13% of the total suspensor DNA.

It should be pointed out here that the cytological evidence obtained by AVANZI *et al.* (1970), and confirmed by NAGL (1973), has been questioned by BRADY (1973) on the grounds of the absence of the nongeometric increases in DNA contents per nucleus expected in the case of selective gene amplification. BRADY'S results, however, do not seem to infirm what has been stressed previously, as they are only based on questionable Feulgen cytophotometric data.

More support for the presence of reiteration processes in differentiated and differentiating cells can be found in the work of several authors (SOSSOUNTZOV, 1969; AVANZI and D'AMATO, 1970; AVANZI and CIONINI, 1971; AVANZI *et al.*, 1974) on other organs and tissues, e.g. *Gingko biloba* gametophyte, the quiescent center of *Calystegia* and apical cells of *Marsilea*.

Particularly interesting and conclusive, however, seem to be the data of PEARSON *et al.* (1974) on differences in redundancy between DNAs extracted from *Cucumis melo* and *C. sativus* tissues. These authors, using CsCl analytical ultracentrifugation, compared the percentages and densities of DNA satellites in seeds, roots, flowers, hypocotyls, leaves, cotyledons, fruits having variation in both parameters. Percentages of DNA satellites varied from 14.1% in cotyledons to 36.3% in seeds and 29.7% in roots, and showed generally higher values in the presence of dividing cells. Moreover, there was a consistent increase in density from seeds to fruits which was considered as a further proof of differential replication preferential for certain sequences within the satellite fraction. Finally a correlation was found between the amount of satellites and the percentage of heterochromatin in the nucleus. The general conclusion of this work was: "that differential replication may be a common though complex phenomenon associated with the differentiation of non-dividing cells", a conclusion which might well be extended to most of the experiments reported in this article.

All the examples we have reported up to now suggest the existence of transient amplification processes during differentiation (elongation, metaxylem) and inspecialized cells (*Phaseolus* suspensor, gametophytes, cells of the quiescent center). We shall now proceed to survey the available data on extra-DNA synthesis in cells reverting from a differentiated to an undifferentiated state.

2.2 Amplification in Dedifferentiating Cells

It is in this field that plant tissue cultures have offered the first test systems in which temporal sequences in DNA synthetic patterns could be studied in a standardized way under environmentally controlled conditions. The first results on DNA amplification during dedifferentiation, however, are derived from knowledge obtained from wound healing experiments mainly carried out to gain insight into the problem of *Agrobacterium tumefaciens* induced tumorogenesis. These studies particularly concern the so called "conditioning" phase i.e. a period between wound and infection with the bacterium necessary for tumorization to occur (BRAUN and MANDLE, 1948). Following an earlier report by KLEIN *et al.* (1953) on increases in DNA during tumor induction, KUPILA and STERN (1961) carried out a thorough analysis of DNA contents in healthy, wounded, and infected internodes of *Vicia faba*, finding a sharp increase in DNA after wounding. This process reached a peak after two days and then decreased in non-infected tissues. This phenomenon was found to be independent from infection and not directly connected with mitoses which only started at the end of the third day of experiment. That the DNA thus synthesized played an important role in tumor induction, was shown later by BOPP (1964) who inhibited tumorization by treating *Bryophyllum daigremontiana* plants before infection with 5-fluorouracil or 5-fluorodeoxyuridine. This inhibition was also found to be reversed only by thymidine and not to be connected with any possible effect on the viability of bacteria. LIPETZ (1967, 1970) further showed the existence of two DNA synthetic waves during *Kalanchoe* wound healing, only the first of which seemed to be necessary for tumor formation and proliferation. DNA synthesis during the first 30 h of wounding was also measured by KUPILA and THERMAN (1971) in *V. faba* stems. In

this case ^{3}H-thymidine incorporation was found to rise above control levels about 10 h after wounding and to reach a peak at 19 h. This pattern was consistent with the minimum transformation time found in *V. faba* and other plants and confirmed the importance of the first wave of synthesis not connected with the mitotic processes hypothesized by LIPETZ (1970) in the above-mentioned work. All these data suggested the need of a specific extra DNA synthesis for cell proliferation after wounding, and prompted QUETIER *et al.* (1969) and GUILLE (1972) to a thorough analysis of the physico-chemical characteristics of this particular DNA. Out of 14 Dicotyledons studied, 10 showed the appearance, 48 h after wounding, of a DNA satellite of buoyant densities varying from 1720 to 1727 g/cm^3. This satellite, which was not found in Monocots, disappeared after 72 h and seemed hence the result of a transient DNA amplification probably essential for cell proliferation (wound healing) and tumor induction. As far as the nature of this DNA species was concerned, the G + C content was estimated to be around 64% and hybridization experiments showed that at least part of the satellite coded for ribosomal RNAs (QUETIER, 1970). Moreover, further experiments seemed to suggest that a certain amount of the sequences studied and amplified after wounding could be complementary to corresponding sequences existing in the *A. tumefaciens* genome (GUILLE, 1972). It should be stressed here that the above-mentioned results have been subjected to criticism by several authors who attributed the presence of high buoyant density DNA species to bacterial contamination. Particularly, SARROUY *et al.* (1973) could stimulate the synthesis of a satellite of bacterial origin through gibberellic acid (GA_3) in radish seedlings, and inhibit it with abscisic acid, thus showing that hormonal treatments can contemporarily influence the metabolism of the plant and of its contaminants.

It seems evident that such possible biases can only be excluded in experiments carried out under strict sterile conditions and in which good correlations can be shown between cytological data, the presence of the first (i.e. localization studies of the synthesized DNA) being essential also for a better definition of the role of the observed amplification phenomena. Plant tissue cultures offer an ideal system for this kind of studies, being sterile and easily maintained under controlled conditions. They also offer the advantage of an easy study of temporal sequences in dedifferentiation events. Again, several reports can be cited where DNA synthesis without mitoses following cutting of tissue portions has been observed during culture of the primary explants. For instance, WATANABE and IMASEKI (1973) studied DNA synthesis through incorporation of ^{32}P orthophosphate and ^{3}H-thymidine in potato slices. Results obtained with both methods suggested a cutting induced net synthesis of DNA with a lag period of less than 6 h, and a peak in the incorporation rate at 24 h after slicing.

Cytological analysis confirmed the nuclear localization of DNA synthesis but failed to show any mitotic event connected with it. These data, together with those of DAS *et al.* (1958) obtained on excised tobacco pith tissue, strongly suggested a specific action of wounding (in the absence of hormones in the culture medium) on non-mitotic DNA replication. Similar results have been obtained later, with the use of 2,4-dichlorophenoxyacetic acid (2,4-D) in Jerusalem artichoke tuber tissue by YASUDA *et al.* (1974). In this case ^{3}H-thymidine incorporation was observed only when the hormone was added to the medium, increased rapidly

after 20 h from the initial explant and reached a maximum at 36 h, decreasing thereafter. It is worth noting that in both experiments reported above RNA and protein synthesis inhibitors also inhibited DNA synthesis, thus suggesting a phase, possibly coinciding with the lag period, of metabolic activation prior to DNA replication.

In this kind of studies, however, little attention was paid to the nature of the DNA synthesized and/or its cytological localization. With the purpose of obtaining a better insight into this side of the problem, NUTI RONCHI and GREGORINI (1970) carried out a histological and cytological study of the dedifferentiation processes in *Lactuca sativa* cotyledons grown in vitro. This material seems particularly suitable for such a study because of the high nuclear homogeneity of the starting material (only 2C cells are present according to BRUNORI and D'AMATO, 1967). In this work a transient appearance of extranuclear Feulgen positive bodies during the first days of the culture was observed.

Cytochemical studies carried out later supported the presence of DNA in those bodies, while ^{3}H-thymidine incorporation experiments showed a clear localization of the label in the perinucleolar regions (NUTI RONCHI, 1971). All these phenomena occurred before cell division, thus suggesting the presence of gene amplification processes essential for further proliferation and differentiation (lignification) to occur.

Better results, however, were obtained on the same line of research using another and more suitable dynamic test system, the *Nicotiana glauca* pith tissue (NUTI RONCHI *et al.*, 1973; MARTINI and NUTI RONCHI, 1974). The advantages of this test were the absence of the heavy chloroplast concentrations found in *Lactuca* and the histological homogeneity of this material when freed from surrounding vascular tissue.

Pith explants were excised, cultured on a 2,4-D containing medium and cytological events followed during the first 144 h of culture. In the period from 48 to 96 h of culture a very high percentage of cells showed within the nuclei a massive formation of Feulgen positive bodies resembling nucleoli, which later were extruded into the cytoplasm. At the same time the nuclei became swollen and lobed and a disintegrating nuclear membrane was often observed. Later on nuclear fragmentation and the consequent formation of polynucleate cells (an average of 4–5 nuclei per cell with 20 nuclei peaks) were reported, which eventually led to the organization of meristematic centers. ^{3}H-thymidine experiments showed the presence of localized labeling in the perinucleolar region and in the Feulgen positive extruded bodies around 48 h after the primary explant. On the other hand the label was extended to the whole nuclei at later hours prior to the initiation of a mitotic cell division wave.

More support to the hypothesis of gene amplification preceding proliferation was derived from further microdensitometric data obtained on the same material (MARTINI and NUTI RONCHI, 1974). Two concomitant processes were suggested to occur on the grounds of the information gained through the use of this technique: an extensive amplification process restricted to the period between 48 and 120 h of culture and a total DNA reduplication which maintained the nuclei at higher DNA content even at later times. This hypothesis was mainly derived from the observation of a nongeometric increase in DNA contents during the "amplifi-

cation" period, followed by a reduction due to nuclear fragmentation and a return to values falling into a normal geometric series. DNA values per nucleus in polynucleate cells were inversely proportional to the number of nuclei, thus giving a clear proof of the nuclear fragmentation process. The cytological and cytochemical results reported above prompted a detailed biochemical study of the DNA(s) synthesized after different periods of culture; this was carried out by analyzing extracted DNA through CsCl analytical ultracentrifugation (PARENTI *et al.*, 1973). A heavy DNA satellite (density 1722 g/cm^3) was thus shown to appear, to increase in amount steadily up to 72 h and then decrease later; an additional satellite was also observed at 1705 g/cm^3 and found to be constantly present whatever the period of culture. Hence the formation of the extra DNA species seemed to parallel, in its temporal sequence, that of nucleolar extrusion and localized synthesis observed at the cytological level. Moreover, it could not be attributed to bacterial contamination due to the negative results of sterility tests carried out on homogenates of the tissue used for DNA determinations. Further support for a dynamic DNA heterogeneity during pith dedifferentiation was given by thermal denaturation profile analysis, which showed the presence, during the period of DNA satellite synthesis, of an extra DNA family, presumably of high G+C content (DURANTE *et al.*, in preparation). As to the nature of the extra-DNA, preliminary hybridization experiments carried out with ^{3}H-uridine labeled ribosomal RNA showed only a 30% increase in r-DNA multiplicity during the amplification phase, thus suggesting that part, but probably not all of the sequences involved in the process coded for r-RNA. Finally the question whether or not the extra-DNA synthesized was really a prerequisite for cell proliferation was tackled through the use of specific inhibitors during and after the amplification period. Particularly interesting are, in this respect, the results obtained through the use of BUDR (DURANTE *et al.*, in preparation), which was shown to inhibit growth and callus formation only when given during the extra-DNA synthetic period. After 48 h of culture, the analog was found to be specifically incorporated into a DNA portion whose buoyant density shifted to 1738, the main band remaining at 1695. After 144 h, however, the satellite, as mentioned before, disappeared and a large DNA peak (1713 buoyant density) appeared, thus showing BUDR incorporation into nuclear DNA. Cytological analyses of the same material showed only partial inhibition of micronucleoli formation but also a strong inhibition of nucleolar extrusion in samples treated with BUDR in the critical hours.

Results similar to those outlined above have been obtained on other *Nicotiana* species and hybrids such as *N. langsdorffii*, *N. bigelovii quadrivalvis*, *N. bigelovii bigelovii* and a nontumorous mutant of the amphidiploid hybrid *N. glauca* × *N. langsdorffii* (BUIATTI *et al.*, 1974; NUTI RONCHI and MARTINI, unpublished), although less detailed evidence is available in these cases. It should be noted here, however, that proliferation in pith tissue of the tumorous hybrid *N. glauca* × *N. langsdorffii* seems to occur mainly through mitoses without extra-DNA synthesis, as shown also by the lack of a DNA satellite which is present in the nontumorous mutant of this stock (BUIATTI *et al.*, 1974). These, in fact, seem to be the first data reported in the literature of genetic control over amplification capacity, a problem which certainly deserves more consideration in view of the fact that strong genetic

differences in proliferation capacity have been found in several plant species, which hence could offer good material for extensive studies of the problem (BUIATTI *et al.*, 1974; BARONCELLI *et al.*, 1974). A certain kind of hormonal control, resembling that described in the *Cymbidium* seems also to be suggested by several observations. First of all, amplification in *N. glauca* seems to be less striking, and proliferation through mitosis more frequent, in the presence of vascular tissue and in young and vigorous plants, whereas the opposite situation can be observed in individual plants near to the flowering stage and in vessel-free explants. Moreover, a certain seasonal dependence of the phenomenon has been observed which might derive from varying temperature and light conditions and their influence on the general physiological situation of the test plants (NUTI RONCHI and MARTINI, unpublished).

2.3 The General Nature of Transient Amplification

The data so far reported clearly support the hypothesis of a widespread occurrence of dynamic DNA amplification phenomena in different phases of plant development. Extra DNA synthesis has been observed or suggested in a variety of differentiating, differentiated and dedifferentiating cell systems; these observations allow the formulation of the working hypothesis that selective replication might be one of the main control mechanisms in plant growth and morphogenesis. This process has been often found to be under hormonal control and a few data suggest that genotypes may also exert an important influence on it. Timing experiments, based mainly on plant tissue culture test systems, seem to have definitely proven the labile nature of this kind of DNA, placing its permanence-time in the cells in the range of hours or a few days. This kind of result certainly gives new value to earlier reports (SAMPSON *et al.*, 1965; SAMPSON and DAVIES, 1967; ANKER *et al.*, 1971; HURST *et al.*, 1971; MACLEOD, 1973), which suggested the existence of a particular DNA species called "metabolically labile", whose synthesis and degradation were found to occur during differentiation of systems like collenchyma (SAMPSON *et al.*, 1965), ageing internodes (ANKER *et al.*, 1971), roots (HURST *et al.*, 1971; MACLEOD, 1973; SAMPSON and DAVIES, 1967). Only a few data are however available on the problem of the nature and coding properties of extra DNAs, although a few instances have been reported of the involvement of ribosomal DNA cistrons (QUETIER *et al.*, 1968; CHEN and OSBORNE, 1970; AVANZI *et al.*, 1972).

3. Semipermanent Amplification

The data reported in the preceding Section pose several questions, important both from a theoretical and a practical point of view. Firstly, it remains to be ascertained whether DNA amplification is always transient or could, under certain conditions, be made permanent. This in turn has some practical bearing, given the hypothesized importance of selective replication for plant growth and

development. If reiteration of specific portions of plant genomes increases rates of growth and of protein synthesis, the permanence of these situations over longer periods of time could be of importance in altering developmental behaviors and, in general, parameters relative to "amount of growth" this being certainly relevant to plant selection and breeding. Two known processes (gene compensation and magnification), both observed in *Drosophila*, can be likened to this kind of behavior. The first concerns developmental increases in the multiplicity of genes in one chromosome in the presence of a deletion in its homologue, and has been thoroughly studied for ribosomal cistrons, in the case of the absence of a single nucleolar organizer segment or an entire wild type NO chromosome. In this instance, TARTOF (1973) found an amplification up to a multiplicity optimal for the analyzed strain (e.g. 250 rRNA cistrons amplified to 400) in the X chromosome of somatic cells in both males and females. To the second belongs the reversion, observed by RITOSSA (1968), from the bobbed (bb) phenotype to the normal which was found to take place with an increase in rRNA cistrons. This magnified condition can be inherited, its stability being dependent upon the association of the magnified chromosome with the other nucleolar chromosome. Although both these phenomena seem to provide an answer to artificially altered genetic situations, they nevertheless represent well-studied instances of semipermanent gene reiteration.

Gene compensation and magnification have also been looked for in plants, although not always with success up to now. PHILLIPS *et al.* (1974) failed to detect any evidence of this kind of process in tests carried out using maize stocks monosomic for chromosomes known to carry r-DNA cistrons. FLAVELL and SMITH (1974a, b), on the other hand, found in wheat that, whereas doubling of a SAT chromosome (1 B) increased by 40% the estimated number of rRNA genes, its elimination in nullisomic stocks only decreased it by 15% to 23%. This fact was interpreted as a possible indirect evidence of amplification in other chromosomes of the homologous group 1.

A similar process has been found to occur, although in the opposite direction (r-DNA underreplication), by TIMMIS *et al.* (1972) in the hyacinth. In this case it was shown that the number of r-DNA cistrons was 20% less in aneuploids like $2x+1$, $3x+3$ and $4x-2$, all containing 3 NORs, than in the regular triploid. Well documented amplification processes in plants have however been reported and seem to be bound to specific environmental influences. This is particularly the case for environmentally induced variants in flax and in *N. rustica.*

In 1962 DURRANT first showed that heritable changes in plant weight can be induced in the flax variety Stormont Cirrus, by growing the plants in a moderately heated greenhouse in soil compost to which were added different combinations of fertilizers in solution. Nitrogen in particular given as ammonium sulphate, induced a variant ("genotroph") named L, which is 3 to 6 times larger than the corresponding type (S) induced by phosphorus given as triple-superphosphate. Similar results were obtained three years later by HILL (1965), who grew three highly inbred varieties of *N. rustica* with eight different combinations of nitrogen, phosphorus, and potassium obtaining large heritable differences in flowering time and final height. Further work on flax showed that the developmental changes induced by nitrogen and phosphorus treatments were associated with a difference

in DNA content, as measured with microdensitometric methods of 16% between L and S, the P 1 (plastic) type having 10% less DNA than L and 6% more than S. This particular change was followed in time and found to reach its maximum within five weeks from sowing, with a critical period lasting from the second to the fifth week. This seemed to be the first demonstration of a permanent amplification process in plants and prompted thorough analyses both from the genetical and biochemical points of view. Firstly, a nuclear and a cytoplasmic factor were found to be needed for the plastic characteristic (amplification capacity) to appear, in crosses between a plastic flax variety, Stormont Cirrus, and a non-plastic linseed variety, Royal (DURRANT and TIMMIS, 1973). Moreover, the induced effects were shown to be highly pleitropic and to cover characteristics like number of hair in capsules, plant weight, activity, and relative mobility of peroxidase and esterase isozymes (FIELDES and TYSON, 1973). Careful genetic studies (DURRANT, 1974) have shown, however, that the association between at least three of the above-mentioned characteristics (septa hairness, DNA amount, plant weight) was not complete. This was particularly true in crosses which destroyed or reverted the induced L and S plant weight differences, e.g. those with varieties like Percello, Lyral Monarch, Stormont Motley and Dakota (DURRANT, 1972) and which implied the presence of influences of one chromosome on its homologue in a way similar to those found in *Drosophila melanogaster*. A formal geneticeal analysis, however, seemed rather difficult because of the abnormal behavior of the induced characteristics when studied in the first generation of crossing with normal genotypes. This was particularly the case with the hair character (H-h) (MCLELLAN and DURRANT, 1973), where the heterozygote was found to be unstable and to segregate in F_2 into three heterozygous classes (I, II, III) with approximately 30, 40, 50 hairs per septa.

Further evidence of incomplete association between the DNA increase and other characteristics is derived from the fact that the first could revert to normal values without the others being affected in an appreciable way. This process was found to occur after two generations when plants were grown for five weeks after sowing at temperatures lower than normal.

Biochemical analyses of the DNA in L and S genotypes have also not been able as yet to clarify completely the real nature of observed amplification, although recent data seem to open interesting avenues of research (CULLIS, in press). Neutral CsCl equilibrium centrifugation resolved flax DNA into a main band of 1698 g/cm^3 buoyant density, a less dense satellite (1688–1689 g/cm^3), and a dense shoulder (1709 g/cm^3) (CULLIS, 1973, 1975; TIMMIS and INGLE, 1973).

No significant differences were found between the L and S genotypes as far as the distribution of these three fractions was concerned. Cytological studies showing a 2-fold difference in nucleolar mass between the two genotrophs suggested rRNA-DNA hybridization experiments which indeed allowed the estimate of a difference between L and S nuclei of about 60% in r-DNA cistrons multiplicity (TIMMIS and INGLE, 1973).

Further studies carried out by CULLIS (in press), although reporting a similar (70%) difference between the extreme types showed that r-DNA multiplicity, was the same in DNAs extracted from L and PL genotrophe. Hence, it was concluded

that no amplification for r-DNA cistrons could be envisaged in S but rather a reduction (under replication). In any case, as already stated by TIMMIS and INGLE (1973), a 70% difference could only account for a 0.23% increase in DNA, an amount which would have been negligible when compared with the reported value of 16% difference between S and L. The absence of gross differences in basic composition between the genotrophe was confirmed by the same authors, also through the analysis of differential melting curves, which clearly showed the existence of the same four main types of sequences varying in average basic composition in L, PL, and S.

Better results, after an early failure (TIMMIS and INGLE, 1974), were obtained when renaturation kinetics was studied by CULLIS (in press) with the aim of obtaining estimates of the amount of repeated sequences present in the three types.

Hydroxyapatite fractionation allowed the observation of an intermediate DNA fraction present only in the L genotrophe, and comprising about 13% of its DNA. Moreover, a fast renaturing fraction was found to amount to 29% of total DNA in S and PL, while being only 24% in L. Optical renaturation curves gave similar estimates (respectively 30% and 26%), thus suggesting a dilution of this fraction in L which could account for the observed results if no extra DNA occurred in the fast renaturing fraction. The lack of differences between PL and S DNAs was attributed to a parallel 6% reduction in S in both slow and fast renaturing fractions.

A less advanced analysis than that just described for flax has been carried out up to now with *N. rustica*. The genetic data obtained so far (biochemical analyses are lacking) do not seem, however, to offer a very different picture. EGLINGTON and MOORE (1973) confirmed the instability of F_1s between different genotrophs observed in flax and could follow its permanence up to the fifth generation. This kind of segregation could be distinguished from a normal one which started in the F_2 and revealed the existence of at least 13 factors involved in the differences observed (PERKINS *et al.*, 1971; EGLINGTON and MOORE, 1973; MOORE and EGLINGTON, 1974).

The data available in the literature and described above concerning semi-permanent amplification offer a rather confusing picture, firstly because it is still difficult to attempt any generalization in a process on which direct or indirect evidence only exists for 3–4 plant species, and secondly because the biochemical nature of the amplified portions is still by no means ascertained. As stated by CULLIS (in press) the induction of flax genotrophs could well involve an increase in specific unique sequences in the case of L, a decrease in ribosomal cistrons in S, or a more generalized loss in both repeated and unique sequences in the case of L and S. Moreover, it is still a matter of investigation whether the observed DNA increases are, or are not, the cause of induced changes in developmental behavior. Finally, the mechanism by which DNA amplification occurs remains completely uncovered.

Plant tissue cultures could be very useful in clarifying at least some of these processes, as they might offer the necessary system for studying the possibility of prolonging amplification phenomena already observed and described in this review, through the alteration of environmental conditions of culture. Moreover,

correlations between DNA increases and growth could be studied in the complete absence or presence of differentiation under standard conditions. Developmental plasticity of plant tissue cultures could then allow to induce new types by simply differentiating cultures after specific alterations of the cultural environment.

4. Differential Redundancy, Selection and Evolution

Wide variation in the relative proportions of different DNA species both at the inter- and intraspecific levels have been reported by several authors. The available data, which we shall study later, clearly point to the possibility, already stressed in the preceding part of this review, of a persistence, through meiosis, of situation, of over- or under-replication of specific DNA sequences, or even of their induction during meiosis itself. This might be the case if the DNA synthesis observed during meiosis (FLAVELL and WALKER, 1973; SMITH and STERN, 1973) in several plant species, and in one case found to be selective for repeated sequences (SMITH and STERN, 1973), could be attributed at least in part to amplification processes and not only to repair events connected with crossing over. This would put some early results in a new light (SPARROW and HAMMOND, 1947) on extrusion during meiosis of Feulgen positive nuclear material into the cytoplasm of *Lilium henryi*, *Trillium erectum* and *Trillium grandiflorum*.

The bulk of the evidence present in the literature on differential redundancy is mainly derived, however, from studies on variations in percentages of ribosomal genes and/or of heterochromatic and highly repeated portions between and within species, the choice of the parameters being due to the existence of good biochemical and cytological techniques, which allow the discrimination of these kinds of sequences from main DNA.

Intraspecific differences in nuclear DNA content have been reported recently for several conifers like *Picea glauca*, *Picea sitchensis*, *Pinus banksiana*, *Pinus resinosa* and *Pseudotsuga menziesii* (DHIR and MIKSCHE, 1974). In these cases DNA amounts, from the lowest to the highest plant, varied by factors ranging from 1.5 in *P. banksiana* to 2.2 X in *P. resinosa*.

PEARSON *et al.* (1974) in a previously mentioned study on differential DNA replication during development in *Cucumis melo*, found, to their surprise, that the percentage in different tissues of a 1705 g/cm^3 buoyant satellite varied both between varieties or types and within varieties between plants. For instance, in seeds the relative proportion of the satellite ranged from 26.1% in Hero of Locking to 29.6% in Spanish Winter, while the variation between individuals belonging to the last variety was from 24.3% to 36.3%. Similar results were obtained for cotyledons (from 14.1% to 29.9%) and fruits (from 15.2% to 23.9%), the experimental error involved in preparation and analysis amounting only to a 3% spread of values.

Differences in ribosomal multiplicity were found first in maize where PHILLIPS *et al.* (1974) observed a rRNA cistron numbers varying from 4700 to at least 8200 among various agronomically desirable inbred lines. A detailed analysis of a similar situation was carried out later by FLAVELL and SMITH (1974a, b) on seven

hexaploid wheat and two diploid rye varieties. In this study a 2.2 fold variation in r-DNA multiplicity was found in wheat and a 2.5 in rye. Further analysis with substitution lines chromosomes carrying nucleolar organizers suggested an additive action of these chromosomes and no interaction, as far as the rDNA multiplicity was concerned, with other chromosomes of the wheat complement, although the data for one variety were somewhat doubtful. As stated by FLAVELL and SMITH (1974a, b) these results point to the existence in the *Triticinae* of a mechanism for altering rDNA multiplicity. A number of questions are clearly posed by the limited results so far obtained on intraspecific variation in multiplicity. Firstly, nothing is known as yet on the real mechanism of induction of the observed variability, which could occur either through amplification and integration of new DNA copies (RITOSSA, 1972), or through processes like unequal crossing-over (TARTOF, 1973). Secondly, it is rather difficult to ascertain whether the observed differences have been brought about by (unconscious) selection or arise from a process similar to random drift, i.e. random segregation of variants. This is certainly not a trivial question because an answer in favor of selective pressure for or against genomes amplified for certain genes could open large possibilities to nonconventional plant breeding procedures. The first problem which needs clarification in this context is whether differences in multiplicity really mean changes in growth and developmental parameters or not. If this is the case, as selection operates throughout development, and not only at the reproductive stage, it would be easy to envisage the possibility of selective forces acting on multiplicity differences. So far, the only preliminary data available on these problems come from work carried out on hexaploid wheat tissue cultures. With this material, a correlation has been found between callus growth and ribosomal multiplicity when the two parameters were compared in four varieties and four ditelocentric sticks of hexaploid wheat (BUIATTI *et al.*, unpublished). Moreover JAIN *et al.* (1968) found that deletion of one arm of chromosome 1A, which was later observed to bring about a 58% reduction in r-DNA multiplicity, also decreased RNA synthetic activity, thus supporting the hypothesis of a direct relationship between multiplicity and rate of RNA synthesis.

More information, however, on the selective and evolutionary role of differences in redundancy certainly comes from data concerning interspecific variation and generally from studies carried out on an evolutionary scale. Again, data on multiplicity of r-DNA are largely available. MATSUDA *et al.* (1970), analysing tissues from some 19 plants species, found values for rDNA redundancy ranging from 0.08% (Wheat germ) to 0.83% (Chinese cabbage). Specific studies carried out later showed a variation in *Cucurbita* from 1.4% to 3.1% (GOLDBERG *et al.*, 1972), in *Nicotiana* from 0.27 to 0.90% (SIEGEL *et al.*, 1973), in *Vicia* from 0.11% to 0.31% (MAHER and FOX, 1973). In the *Vicia* and *Nicotiana* studies a lack of correlation has been claimed between DNA contents and r-DNA amounts, which suggested the possibility of changes in the relative proportions of certain genes during evolution. This was not the case in a polyploid series of *Datura* produced through in vitro culture of pollen grains, where a direct proportionality between ploidy and r-DNA cistron number was found to occur by CULLIS and DAVIES (1974). CULLIS has also recently failed to repeat the data obtained on *Nicotiana* by others (CULLIS, personal communication). Variation in DNA satellite contents

has been extensively studied by INGLE *et al.* (1973), who found differences both in the bouyant densities and in percentage of total DNA in species belonging to 17 dicotyledon families.

A more precise evolutionary role for differential redundancy has been suggested by NAGL and EHRENDORFER (1974) after a series of experiments on a group of *Anthemidae* (Asteraceae). In this work a comparison was carried out between 12 annual and 6 perennial species using parameters like DNA content, nuclear volume, mitotic index, cell cycle duration, and amounts of heterochromatin. Annuals were thus found to have more heterochromatin than related perennials, a finding which correlated well with earlier data by MIKSCHE and HOTTA (1973) on increase of repetitive DNA with increasing DNA contents.

NAGL (1974) was further able to find a correlation between relative amounts of heterochromatin and cell cycle duration. All these data suggested an evolutionary significance for heterochromatin which, according to NAGL (1974), could be one of the factors which influence cell cycles and through it the rate of development in plants. In this way annual species could reach a higher DNA level without harmful increases in cell cycle and generation time.

5. Concluding Remarks

It seems clear that all the evidence reported in this article points to the existence of a wide genetic (in terms of DNA sequences) variability in plants not directly connected with mutations in structural genes. This kind of variability is due to quantitative changes in the amount of information rather than qualitative ones, like those induced by mutations. These changes can be induced at several stages of plant development and probably in a specific way, when drastic changes in developmental canalization patterns are brought about by changing internal (physiological) or external (environmental) stimuli. Developmental, temporal patterns of these processes can in fact be followed in systems, like plant tissue cultures, which can be observed under standard conditions and where phase changes can be induced with known timings through modification of one or few environmental variables. The nature of the DNA sequences subject to these alterations remains, however, largely unknown, although it can now be said with a good degree of certainty that they do not affect only well-defined classes of cistrons like those coding r-DNA. It is also not clear whether amplification phenomena can be made permanent by altering the cellular environment, a subject for which tissue cultures can again be useful and which deserves more consideration.

The importance of all these processes for plant breeding is derived mainly from the evidence presented in the last part where differences in relative multiplicity were correlated with variation in developmental patterns. If this holds true, as is suggested by the data so far available, genetic engineering could include induction of permanent increases or decreases of regulatory sequences (repetitive DNA), or of reiterated cistrons necessary for the prevalence of a certain kind of canalization, given of course a detailed knowledge of the mechanisms of induction of amplifica-

tion. Alternatively, selection could be carried out, as it probably has been on an evolutionary scale, for improvements in the relative ratios of cistrons of known regulatory function. This would be particularly easy if a direct correlation between in vitro growth and differentiation parameters on the one hand, and redundancy for particular cistrons on the other, could be really proven, as suggested for wheat (BUIATTI *et al.*, unpublished).
Generally speaking, it can certainly be said that the possible impact of such new techniques, and of the real meaning of quantitative regulation via differential replication, are still matters of hypothesis. A good part of the questions posed by the data reported can now, however, be tested better in plants than in animals, given the large plasticity of systems like tissue cultures.

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References see page 442.

3. Applied Aspects of Embryo Culture

V. RAGHAVAN

1. Introduction

The embryo represents the beginning of the new sporophytic generation of the plant. In virtually all the higher cryptogams, the embryo has its origin in the fertilized egg or the zygote, which is generally buried within a privileged and relatively inaccessible location in the female gametophyte. In seed-bearing plants, it is not unusual for accessory embryos to arise from cells other than the zygote. Embryos of different developmental stages, formed within the female gametophyte through the normal sexual processes, can be separated with relative ease from the bulk of the maternal tissues and cultured in vitro under aseptic conditions in media of known chemical composition. This approach has tempted investigators from time to time to study the factors which influence the nutrition, growth, and differentiation of embryos of several angiosperms, gymnosperms, and pteridophytes as they grow outside the environment of the ovule and are nurtured by exogenous nutrients. Advances in embryo culture method have served to open the way effectively to obtain plants from inviable hybrids, the seeds of which are traditionally condemned and discarded due to their inability to germinate under normal conditions. In some types of seeds, which germinate only after a period of storage, dormancy can be overcome by excision and culture of embryos, and treatments generally used in overcoming dormancy in other seeds are ineffective. In still other types of seeds, whose dormancy is broken by specific light or temperature treatments, embryo culture has helped to localize the endogenous promotors or inhibitors of germination which presumably maintain the seeds in the nondormant or dormant state. Results of studies on the culture of embryos of root and stem parasites and of seeds with underdeveloped embryos have provided valuable clues to their varied morphogenetic potentials. Embryo culture method thus offers a new and refined parameter to characterize embryogenesis and related problems in plants.

The intent of this article is to review the possible practical applications of the techniques directly related to the isolation and culture of embryos. Since a comprehensive coverage of this information is admittedly difficult within the confines of a single article, examples are chosen in an attempt to give an idea of the range of plants studied and an appreciation of the problems involved. The account will, however, begin with a brief introduction to the technique of culture of embryos.

2. Technique of Embryo Culture

Some of the methods employed in the excision and culture of embryos of vascular plants are described by SANDERS and ZIEBUR (1963), RAGHAVAN (1967) and TORREY (1973). Aseptic procedures are to be followed in the excision of embryos and their transfer to nutrient medium. This is less forbidding than it sounds; since the embryos are lodged in the sterile environment of the ovule, surface sterilization of the embryos as such is not necessary. Instead, entire ovules,

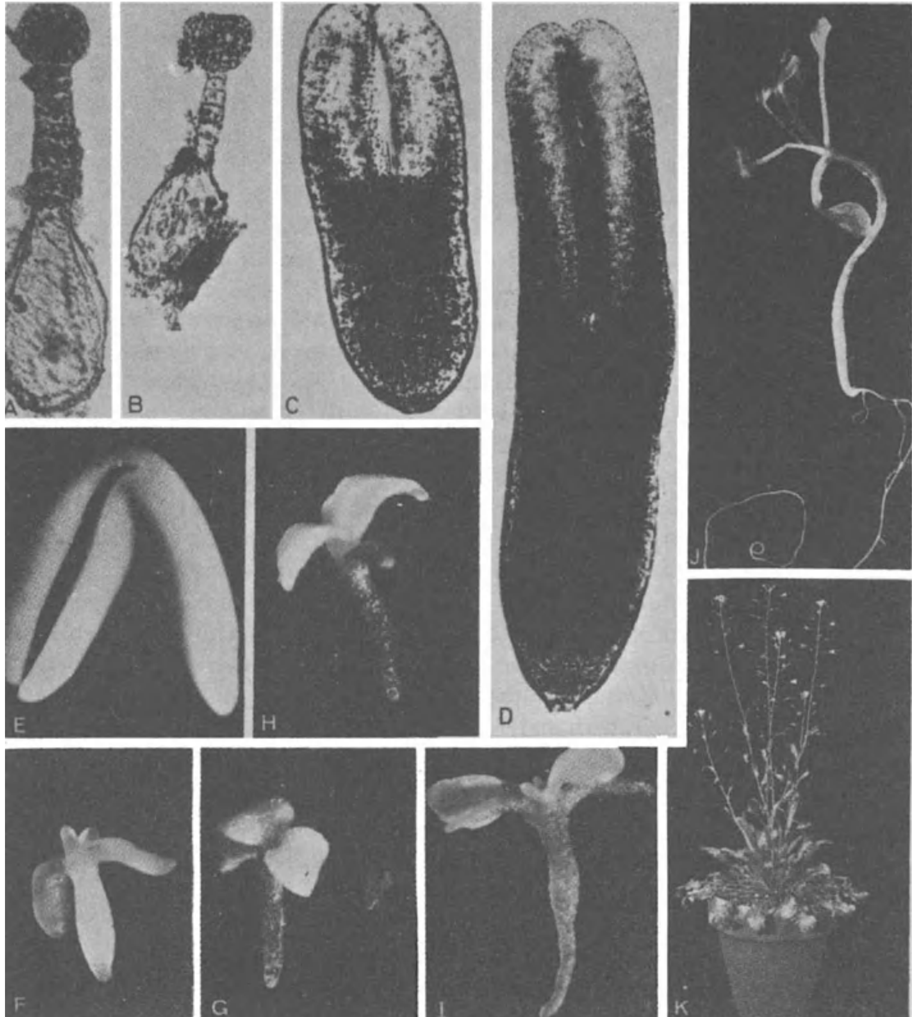


Fig. 1A–K. Stages at excision and growth in culture of embryos of *Capsella bursa-pastoris*. (A–E) Whole mounts of embryos; (A) Early globular embryo; (B) An early heart-shaped embryo; (C) An intermediate stage embryo; (D) A torpedo-shaped embryo; (E) Nearly mature inverted-U-shaped embryo (after RAGHAVAN and TORREY, 1963); (F–K) Various stages in the formation of a normal plant from a cultured torpedo-shaped embryo

seeds or capsules containing ovules are sterilized and the embryos aseptically freed from the surrounding tissues. With hard-coated seeds, isolation procedures begin with soaking them in water for a few hours to a few days so that the embryos can be removed with ease. Seeds are generally surface-sterilized before soaking, although after prolonged periods of soaking they may need to go through a second sterilization.

Splitting open the seeds and transferring embryos to the nutrient medium is the simplest technique that can be used with seeds. Procedures for the isolation of comparatively smaller embryos require that they be removed intact from the ovule without damage; this aim can best be achieved by carrying out the operations under a dissecting microscope.

Of particular interest are the seeds of orchids which contain morphologically undifferentiated embryos corresponding to the globular embryos of dicotyledons. There are no functional storage tissues in the seed such as the endosperm to interfere in nutritional studies, and the seed coat is reduced to a membranous structure. For these reasons, the entire ovule of the orchid containing an undifferentiated embryo is cultured and treated as embryo culture (RAGHAVAN and TORREY, 1964). In addition, the relatively small size of embryos makes cultivation of large samples possible.

The most important aspect of embryo culture work is the selection of the medium necessary to sustain continued growth of embryos. Although the nutrient media used to culture embryos are as varied as the number of species studied, it appears that the younger the embryo, the more complex is its nutrient requirement. Thus, while relatively mature embryos can be grown in an inorganic salt medium supplemented with a carbon energy source such as sucrose, relatively small proembryos require, in addition, different combinations of vitamins, amino acids, growth hormones, and natural endosperm extracts like coconut milk. Since proembryos are embedded in the ovular sap under considerable osmotic pressure, culture of such embryos in an osmoticum such as mannitol, is also recommended. After the embryos have grown into plantlets in vitro, they are removed from the original medium, and nurtured in sterilized soil or vermiculite and grown to maturity in the greenhouse. Stages in the formation of a plant from cultured embryos of *Capsella bursa-pastoris* are illustrated in Figure 1.

3. Culture of Embryos of Inviable Hybrids

Before attempting to describe the application of embryo culture method in overcoming inviability in plants, it is advantageous to examine briefly the causes for this anomaly. In horticultural and breeding practices, embryo abortion is normally encountered in the seeds of unsuccessful crosses. Although fertilization occurs normally in such crosses, and embryos begin to develop in a relatively normal way, a number of irregularities subsequently set in, resulting in the eventual death of embryos and collapse of seeds. Part of the problem in this section is to discern the kind of disturbances that occur and to determine how they modify the normal developmental processes in the complex setting of the ovule, resulting in embryo abortion.

3.1 Development of the Embryo and Endosperm

Embryo Development. There are some close parallels in the pattern of embryo development seen in a number of different inviable matings, and virtually all investigators seem to agree in identifying an early embryonic stage when developmental anomalies begin to surface. A type of abnormal development that may be taken as sufficiently representative was described by RENNER (1914) in reciprocal crosses between *Oenothera biennis*[1] and *O. muricata*, and between *O. biennis* and *O. lamarckiana*. Here the initial rate of growth of the embryo was not appreciably affected, but growth slowed down later and eventually, in most cases, did not advance beyond a few-celled stage. Development progressed further in some afflicted ones, but survival was generally erratic and ovules harboring underdeveloped embryos matured into the more familiar shrunken aborted seeds. The em-

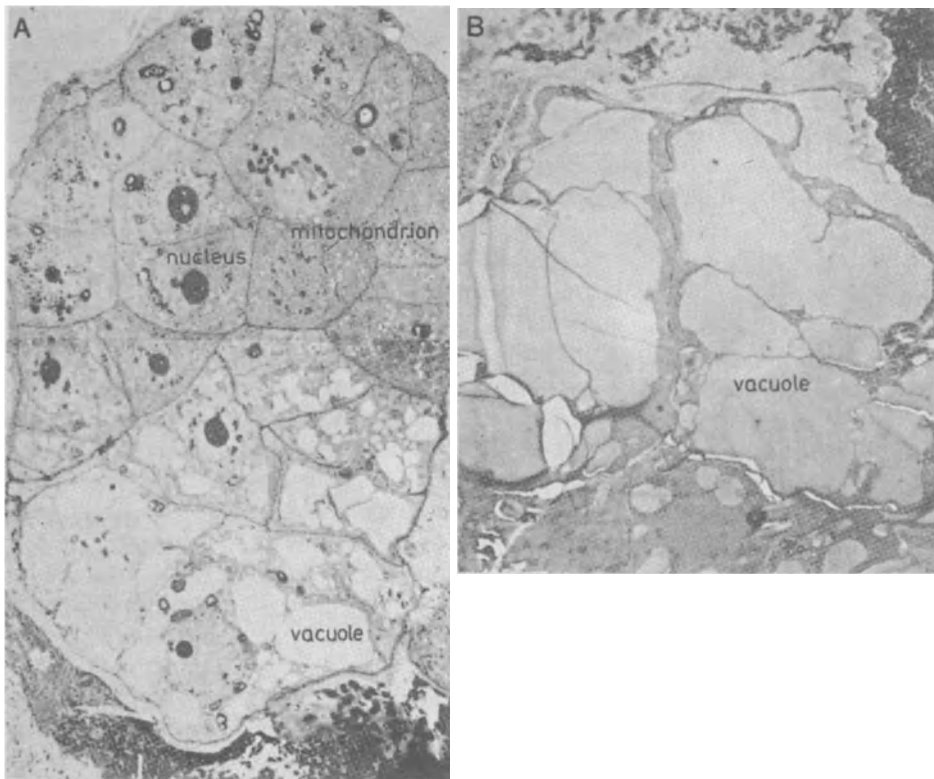


Fig. 2A and B. Ultrastructure of embryos of selfed *Hibiscus costatus* (A) and *H. costatus* × *H. furcellatus* hybrid (B) at the mid-globular stage of development. Vacuoles are more abundant in the basal cells than in the apical cells of the selfed embryo. Note the paucity of organelles in the cytoplasm of the hybrid embryo. (After ASHLEY, 1972; photograph kindly supplied by Dr. TERRY ASHLEY)

[1] In designating crosses, the stock used as the female parent is written first.

bryological history of crosses between different species of *Datura* also illustrates similar developmental failures in hybrid embryos (RIETSEMA and SATINA, 1959).

Recently, ASHLEY (1972) has examined the ultrastructural differences in early embryo development between selfed *Hibiscus costatus* and *H.costatus* × *H.aculeatus* and *H.costatus* × *H.furcellatus* hybrids. After fertilization, the zygote of the self-fertilized parent underwent an abrupt or, less frequently, a gradual decrease in size to about 50% of the volume of the unfertilized egg. Such a shrinkage in size was also marked by a pronounced segregation of the cytoplasmic organelles at the chalazal end marking the beginning of polarity. There was no appreciable shrinkage in the hybrid zygotes, which also failed to undergo the sequence of polar organization of the cytoplasm that normally occurred. Generally, the hybrid genome appeared to have a lower synthetic capacity as indicated by the paucity of organelles in the cells and vacuolization of the cytoplasm. The result of these changes was that the hybrid embryos formed no more than a clump of highly vacuolate necrotic cells, and aborted by the time the normal embryo had reached the globular stage; the cells of the aborted embryo rarely bore even the most tenuous resemblance to the normal embryo (Fig. 2).

In white pines [subgenus *Strobus* (Haploxylon)], embryo inviability is a barrier of major importance to crossability, although in hard pines [subgenus *Pinus* (Diploxylon)] hybrid failures are due to pollen incompatibility (MCWILLIAM, 1959; KRIEBEL, 1972). Extensive observations of the sterile crosses *Pinus peuce* × *P.cembra*, *P.peuce* × *P.koraiensis*, *P.strobus* × *P.flexilis*, *P.strobus* × *P.cembra* and *P.strobus* × *P.koraiensis* have shown that, although fertilization takes place normally, the embryo degenerates inside the archegonium or when the suspensor tiers elongate and thrust the apical unit into the female gametophyte (HAGMAN and MIKKOLA, 1963; KRIEBEL, 1972). In summary, these results clearly indicate that embryos of inviable hybrids possess the potential for development, but are somehow prevented from reaching the adult size and character in their full multicellularity and structure. In certain crosses, disorganization of the embryo following successful pollination and fertilization is due to self- and cross-sterility (BRADBURY, 1929; BRINK and COOPER, 1939, 1940).

Endosperm Development. Considering the nutritional dependence of the embryo on the endosperm, it is understandable that the extent to which the latter may develop and persist in infertile hybrids has attracted much attention. To RENNER (1914) must be given the credit for the first clear account of endosperm development in an unsuccessful cross. He showed that the collapse of the embryo in interspecific crosses in *Oenothera* was preceded by the disintegration of the endosperm beginning soon after fertilization, thus depriving the embryo of the immediate source of food supply. This basic observation on endosperm behavior has been verified in sufficient detail in several crosses. COOPER and BRINK'S (1945) cytohistological observations of ovules resulting from matings involving *Lycopersicon pimpinellifolium* as the female parent have provided information about the comparative growth of the endosperm in viable and inviable hybrids. As seen from Figure 3, the first one or two rounds of division of the endosperm nucleus in the selfed plants and hybrids were synchronous, but afterwards the endosperm of the selfed plant dramatically outstripped the others in its growth.

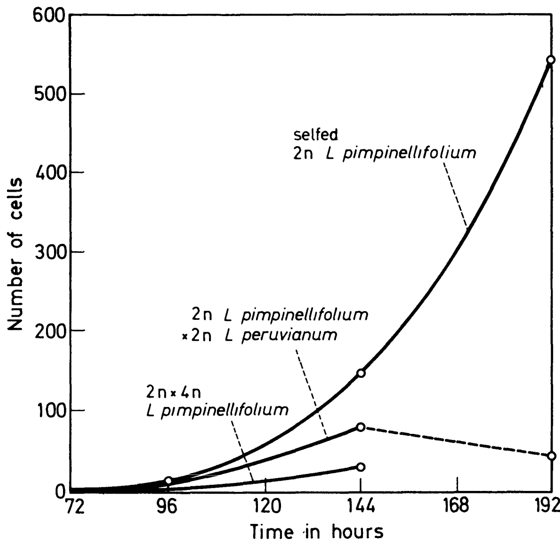


Fig. 3. Number of cells present in the endosperm of crosses between *Lycopersicon pimpinellifolium* and *L. peruvianum* is plotted against time after fertilization. (After COOPER and BRINK, 1945)

At about 144 h after pollination, cells of the endosperm at the chalazal end of the infertile hybrid became vacuolate and less dense than those at the micropylar end. Although the endosperm survived for some time more, it underwent no further divisions; instead, as a result of dissolution of the cell walls and fusion of the protoplasts and nuclei with those of contiguous cells, the endosperm became reduced to a few giant multinucleate, cytoplasmic enclaves surrounding the embryo, simulating the characteristic syndromes of cytopathologic changes. Embryo degeneration may commence when the endosperm is still healthy, or along with or after the disappearance of the endosperm. The extent to which disturbances in the endosperm in an inviable cross make it incapable of supporting the growth of the embryo remains elusive indeed, although a frequent correlation of embryo abortion with the onset of endosperm deterioration observed in many plants indicates that it is due, in part, to the activity of the latter. It does not, however, eliminate the possibility that endosperm malfunction may be one of the number of events that serve to trigger embryo abortion, or that endosperm behavior may reinforce this process by providing stimulus for implementation of embryo abortion initiated by another cause.

3.2 Causes of Endosperm Malfunction

Role of Antipodals and Embryo. The underlying causes and mechanisms of endosperm malfunction in hybrids remain obscure, but there are a few pointers. Some workers (BRINK and COOPER, 1944; COOPER and BRINK, 1944; BEAUDRY, 1951) have claimed that endosperm failure in certain crosses might result from abnormal behavior of the antipodals, which in their often-postulated secretory role presumably alter the nutrient supply to the endosperm. A point of view has also developed that the presence of a developing embryo weakens the endosperm and

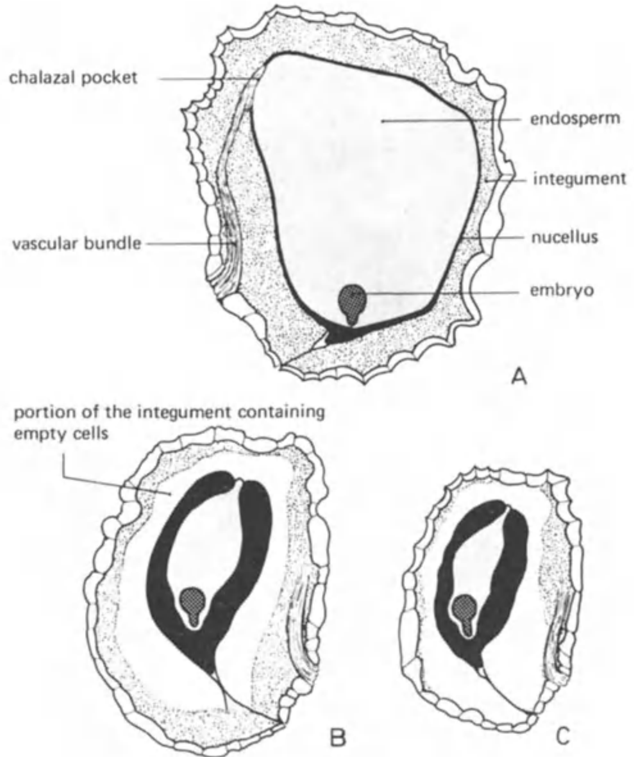


Fig. 4 A–C. Somatoplastic sterility in *Nicotiana*. (A) Section through a selfed *N. rustica* seed. (B) Section through *N. rustica* × *N. tabacum* seed showing nucellar hyperplasie. (C) Hyperplasia in *N. rustica* × *N. glutinosa* seed. (After BRINK and COOPER, 1941)

induces abnormalities in its development. In crosses in *Citrus* involving 2× and 4× parents, it is frequently observed that many embryoless seeds have normal endosperm, while in those containing an embryo, the endosperm invariably degenerates (ESEN and SOOST, 1973). Similar observations have been made in seeds derived from *Gossypium hirsutum* × *G. arboreum* crosses (WEAVER, 1957). It is, however, not known how the developing embryo provokes the inhibition of growth of the endosperm and how the nutritional demands of the embryo are met in the absence of the endosperm.

Role of Somatic Tissues. Abnormalities are known to occur in the development of the somatic tissues of the ovule such as the integuments and nucellus of certain infertile hybrids, and some of these developmental anomalies have substantial interest because of their bearing on the problem of embryo abortion. Although several earlier investigators have observed a relatively weak endosperm development in association with intrusive growth of the nucellus in some interspecific crosses, it was the work of BRINK and COOPER (1941) that presented these observations in an illuminating framework. In *Nicotiana rustica* × *N. tabacum* and *N. rustica* × *N. glutinosa* crosses, most of the hybrid seeds collapsed at various stages of maturity, whereas upon being self-pollinated, *N. rustica* yielded a full complement

of seeds. Seed collapse was associated with a retardation in growth of the endosperm, which attained only a fraction of growth attained in the selfeld series and a pronounced hyperplastic growth of the nucellus (Fig. 4). The term "somatoplastic sterility" is used to describe this type of hyperplasia of the nucellus. At the same time, the integumentary cells lying between the apex of the vascular bundle and the chalazal pocket failed to differentiate into conducting elements, foreshadowing the suppression of nutrient transport to the endosperm. By contrast, seeds formed in selfed *N. rustica* plants were normal in every respect, including the absence of hyperplastic growth in the nucellus. The collapse of ovules which occurs in *Medicago sativa* after self-pollination has also been ascribed to somatoplastic sterility (BRINK and COOPER, 1939).

Several infertile crosses show a type of tumor formation from the inner epidermis of the integument, the endothelium, which, following fertilization, becomes a densely cytoplasmic layer surrounding the endosperm except for a small gap at the chalazal end. The tumor begins to proliferate near the chalaza at the dorsal region of the endothelium, extending into the embryo sac as a voluminous mass of tissue. The cells of the endosperm surrounding the tumor deteriorate progressively, and eventually the embryo is surrounded by the overgrown endothelial tissue (COOPER and BRINK, 1945; BEAMISH, 1955; JOHANSEN and SMITH, 1956; LEE and COOPER, 1958; RIETSEMA and SATINA, 1959). In all these cases, abortion of the already undernourished embryo was presumably hastened by the ingrowth of the somatic tissue and the simultaneous decrease in endosperm growth.

3.3 Culture of Hybrid Embryos

It is clear from the above that, in the absence of an uninterrupted supply of food materials from the endosperm, embryos do not complete their normal developmental program. The unique nutritional relationship between the embryo and the endosperm tends to suggest that the latent capacity of a hybrid embryo for full development may be expressed in an artificial milieu supplied with exogenous nutrients conforming to the composition of the endosperm. The demonstration of the ability of embryos removed from nonviable seeds to grow successfully in culture has amply justified this assumption. This Section summarizes the relevant studies in which the technique of embryo culture was employed to obtain seedlings from first generation hybrids of normally unsuccessful crosses.

Interspecific Hybrids. Early in the development of embryo culture as a research tool, LAIBACH (1925, 1929) demonstrated that embryos of nonviable seeds of *Linum perenne* × *L. austriacum* could be cultured in a nutrient medium and reared to maturity. This study set the stage for a series of later investigations to surmount barriers to crossability in plants whose embryos aborted in the seeds before they germinated, or whose seeds were unable to support development of embryos to maturity.

Attempts made to grow embryos of interspecific crosses of horticultural varieties of deciduous trees which ripen early, but have a low yield of viable seeds, have opened up the field of fruit tree breeding for exploitation and study (TUKEY, 1934; BLAKE, 1939; SKIRM, 1942; LAMMERTS, 1942). Generally, seeds of apple, peach,

pear and plum, which contain abortive embryos, do not respond to the usual after-ripening treatment at low temperature (stratification) and, in such cases, embryo culture is the most promising method to preserve progenies which may have characteristics of horticultural value. In some cases, hybrid plants raised by embryo culture have proved to be superior to those raised by stratification with regard to earliness in flowering and the number of flowers per tree. In *Cerasus vulgaris* × *C. tomentosa* and *Ribes nigrum* × *Grossularia reclinata* hybrids, it was possible to raise a second generation of plants from seeds which do not normally germinate, by growing the aborted embryos under aseptic conditions (KRAVTSOV and KASYANOVA, 1968). Thus, embryo culture might help in commercial fruit-breeding programs and in wide hybridization between unrelated fruit trees by providing a route for seedling selection and by giving a higher percentage of seed germination.

Because of the economic importance of cotton, considerable attention has been paid to hybridization work in this plant. Attempts made thus far to hybridize Asiatic 2N cotton (*Gossypium arboreum*, $2n=26$) with pollen from 4N American cotton (*G. hirsutum*, $2n=52$) have been unsuccessful, and only marginal success has been attained in rearing hybrid embryos to maturity in culture (BEASLEY, 1940; WEAVER, 1958; JOSHI and PUNDIR, 1966). In contrast, embryos of the reciprocal hybrid grow reasonably well on nutrient media and afford promise of forming vigorous seedlings if they were cultured at a stage large enough to be handled (BEASLEY, 1940; WEAVER, 1957). Successful hybrids of the cross between *G. davidsonii* and *G. sturtii* were obtained by rearing embryos in culture (SKOVSTED, 1935).

In breeding work with crop plants, embryo culture method is useful in the production of hybrids endowed with desirable disease-resistant qualities. Cultivated tomato (*L. esculentum*) is generally susceptible to attack by virus, molds, and nematodes, whereas the wild species, *L. peruvianum*, exhibits varying degrees of resistance to these agents. Although fruit development is normal in *L. esculentum* × *L. peruvianum* hybrids, seeds often harbor underdeveloped embryos which do not readily germinate. Hybrid seedlings have been raised and nurtured to the stage of flowering from such seeds by embryo culture (SMITH, 1944; CHOUDHURY, 1955; ALEXANDER, 1956).

In barley, breeding work is directed towards the production of winter-hardy and mildew-resistant plants. Many winter strains of cultivated barley (*Hordeum vulgare* and *H. sativum*) are deficient in winter hardiness and are susceptible to attack by the mildew (*Erysiphe graminis* f. *hordei*), whereas wild barley (*H. bulbosum*) is reasonably winter-hardy and resistant to mildew attack. When embryos of *H. sativum* × *H. bulbosum* hybrid, which failed to grow in the ovule beyond a few days after pollination, were transferred to a nutrient culture medium, they resumed normal growth and formed transplantable seedlings (KONZAK *et al.*, 1951). Interspecific hybrids have also been obtained in crosses between *H. vulgare*, *H. bulbosum*, *H. brachyantherum*, *H. depressum*, *H. jubatum*, and *H. spontaneum* by culture of embryos (MORRISON *et al.*, 1959; DAVIES, 1960). Wild barley is also resistant to spot blotch disease (*Helminthosporium sativum*). To circumvent difficulties involved in directly transferring genes for spot blotch resistance from the wild to cultivated species, interspecific hybrids have been reared by embryo culture be-

tween wild species *(H. compressum* × *H. pusillum, H. marinum* × *H. compressum)* to cross with the cultivated species (SCHOOLER, 1960a, b). Recently, culture techniques have been further modified to raise seedlings from *H. vulgare* × *(H. compressum* × *H. pusillum)* and *H. vulgare* × *H. hexopodium* crosses (SCHOOLER, 1962). In the tomato and barley crosses described above, only a small number of the cultured embryos proceeded to form normal seedlings, a fact which perhaps emphasizes a need for improvement in the composition of the medium. With proper attention to nutritional requirements, the culture of hybrid embryos could be enormously improved to assure a greater survival and, thus, offer a significant impact on the hybridization program.

Rice *(Oryza)* is yet another crop to which embryo culture has been applied to propagate hybrids capable of withstanding unfavorable environmental conditions, offer resistance to diseases and pests, and give high yields. As a rule, in interspecific crosses within the genus, only malformed embryos are produced in otherwise normal grains. NILES (1951) found that when kernels from crosses between cultivated varieties of rice were planted aseptically on a solidified mineral salt medium, embryos grew into transplantable seedlings. Difficulties encountered in rearing plants from interspecific crosses within the genus were also overcome by culturing embryos (BUTANY, 1958; NAKAJIMA and MORISHIMA, 1958; BOUHARMONT, 1961). Although a mineral salt medium containing a carbon energy source such as sucrose or glucose supported the growth of hybrid embryos, in some cases more complex additives such as coconut milk have been included in the medium to induce growth of embryos (LI *et al.*, 1961; IYER and GOVILA, 1964). The efficiency of embryo culture varies markedly with the hybrid combination and the medium employed. The percentage of survival of the seedlings in some crosses was very low due to their failure to withstand transplantation into soil. Transfer of agar-cultured seedlings to a liquid medium before transplantation into soil has led to a marked improvement in their growth potential and survival, and there are optimistic hopes that plants so obtained may form the basis for production of favorable lines of hybrid rice (SAPRE, 1963; IYER and GOVILA, 1964).

Hybridization work with the fiber-yielding crop, jute, involving *Corchorus capsularis*, a lowland form which gives commercial white fiber, and *C. olitorius*, a highland form which yields the stronger red fiber, illustrates the success of embryo culture where all other methods had failed. The need to produce a hybrid combining the favorable qualities of *C. capsularis* and *C. olitorius* has been a major goal of jute breeders, but their attempts were impeded by the early abscission of pollinated flowers, resulting in low fruit-set. In *C. olitorius* × *C. capsularis* cross, low fruit-set was associated with the premature abortion of the embryo, probably due to an impairment of the capacity of the endosperm to grow into a cellular tissue (GANESAN *et al.*, 1957). Some success in increasing the yield of fruits was obtained by using reciprocally grafted plants as parents (SULBHA and SWAMINATHAN, 1959) or by application of IAA to the pedicels of pollinated flowers (ISLAM and RASHID, 1961). When germination of seeds from *C. capsularis* × *C. olitorius* cross appeared difficult, hormone application to the pedicels was combined with culture of embryos in a medium containing yeast extract, IAA, and kinetin to obtain transplantable seedlings (ISLAM, 1964). Promising developments have also been reported in the hybridization of squash (WALL, 1954), cabbage (NISHI *et al.*, 1959)

and okra (PATIL, 1966; GADWAL *et al.*, 1968) that may link the eventual success of the program with culture of embryos or ovules.

Evidence illustrative of the application of embryo culture technique may also be drawn from breeding experiments conducted in the legumes. In interspecific and 2N × 4N crosses in *Trifolium*, hybrid embryos combining perennial habit with forage quality were cultured with considerable success in a simple mineral salt medium (KEIM, 1953; EVANS, 1962). In *Melilotus officinalis* × *M. alba* cross designed to yield a fine-stemmed, leafy sweet clover, low in coumarin (a compound harmful to cattle), embryo culture was the only means by which hybrid plants could be reared to maturity (WEBSTER, 1955; SCHLOSSER-SZIGAT, 1962). Since no systematic attempts have been made for improvement of this crop for use as a green manure, the field lies wide open for hybridization work, with ample reason to believe that now, as in the past, successful hybrids will be reared through the embryo culture route. In recent years, several species of *Lotus*, especially *L. corniculatus* have been increasingly used as forage crops and much interest has centered on the production of hybrids with such characteristics as indehiscent and soft seeds and disease resistance by embryo culture (GRANT *et al.*, 1962; DAVIES, 1963). Viable plants have been successfully raised by culture of embryos from interspecific crosses in other legumes, notably *Phaseolus* (HONMA, 1955), *Medicago* (FRIDRIKSSON and BOLTONS, 1963) and *Lathyrus* (PECKET and SELIM, 1965).

A few attempts made to propagate progenies of unsuccessful crosses in some of our common garden plants are noteworthy. WERCKMEISTER (1934, 1936) showed that malformed embryos of inviable crosses in *Iris*, which would otherwise perish in the ovule, could be grown to the seedling stage in a mineral salt medium containing sucrose. In succeeding years, embryo culture has become the choice method for propagating seeds of rare species and important hybrids of this genus (LENZ, 1954, 1956). Encouraged by the success obtained in *Iris*, other workers have extended the embryo culture approach to produce hybrid seedlings in garden varieties of lily (*Lilium*) (SKIRM, 1942; EMSWELLER and UHRING, 1962; EMSWELLER *et al.*, 1962) and *Chrysanthemum* (KANEKO, 1957). Undoubtedly, these examples represent only a fraction of the enormous number of unsuccessful hybrids attempted every year by nurserymen and breeders, full exploration of this field requires the propagation by embryo culture of progenies of a wider number of crosses, including some so far completely ignored.

Although embryos of gymnosperm genera such as *Pinus* and *Ginkgo* have been successfully cultured in vitro, reports on breeding gymnosperm hybrids by embryo culture are rare. This may be attributed to the long time lag between pollination and seed maturity which makes breeding work exasperating. STONE and DUFFIELD (1950) have described a case of propagating hybrid seedlings from *P. lambertiana* × *P. armandi* and *P. lambertiana* × *P. koraiensis* crosses by planting embryos encased in the gametophytic tissues on a nutrient agar medium. Since both *P. armandi* and *P. koraiensis* are blister-resistant, these attempts represent a significant step towards breeding a disease-resistant line of pines. It is to be expected that successful rearing in culture of embryos completely devoid of the gametophytic tissue (HADDOCK, 1954) will contribute in some measure to a wider use of embryo culture method in hybridization work in pines.

Experimental taxonomy classically has utilized karyotype analysis of interracial and interspecific hybrids, but securing hybrids has been a fundamental roadblock for cytological studies. *Datura*, a herbaceous genus of approximately ten species is a notable example illustrating this problem. Attempts to hybridize *Datura ceratocaula*, a semi-aquatic Mexican species with whorled branches, smooth capsules, hollow stems, and reduced vascular tissues, with any of the other species within the genus have failed because of the formation of abortive embryos in every case when it was used as a parent. By culturing embryos in a medium fortified with cold-sterilized malt extract, MCLEAN (1946) was able to grow to maturity seedlings from eight out of nine possible hybrids involving *D. ceratocaula* as the pollen parent, and one involving *D. certocaula* as the pistillate parent. Embryos from other inviable crosses involving *D. discolor*, *D. innoxia*, *D. stramonium*, and *D. innoxia* and tree *Datura (Brugmansia?)* have also lent themselves to culture (BLAKESLEE and SATINA, 1944; SANDERS, 1950). The success obtained in hybridizing *D. innoxia* with tree *Datura* is significant in view of the controversial taxonomic position of the latter.

Intergeneric Hybrids. Some applications of embryo culture technique arising out of intergeneric hybridization also warrants description in this Summary. COOPER and BRINK (1944) found that, although potentially functional embryos were formed in *Hordeum jubatum* × *Secale cereale* hybrid seeds, they ceased to grow a few days after fertilization and thus did not attain a germinable condition. It was, however, possible to rear plants by dissecting embryos from the aborted seeds and growing them in White's medium (BRINK *et al.*, 1944). By similar techniques, intergeneric hybrids have also been obtained from crosses between different species of *Hordeum* and *Secale*, *Hordeum* and *Hordelymus* (MORRISON *et al.*, 1959), *Triticum* and *Elymus* (IVANOVSKAYA, 1946, 1962), *Triticum* and *Secale* (RÉDEI, 1955), and *Tripsacum* and *Zea* (FARQUHARSON, 1957).

To extrapolate from the results obtained in the laboratory to field application would be premature, but from the above survey it is clear that a systematic program to raise hybrids of specific crops by embryo culture on a large scale should be developed. Considering that the evolution of successful hybrids has obvious pertinence in improving the quality of our agriculture, a more rapid tempo of investigations is called for in this direction. The inherent practical difficulties of excision and culture of embryos on a mass scale can perhaps be overcome by embryo culture in conjunction with induction of adventive embryos. A reasonable assumption here is that any organ of the plant can be induced to form embryos which faithfully reproduce the typical stages of zygotic embryos.

4. Embryo Culture and Seed Biology

Seed biology is a vast area of study with ramifications into such diverse fields as ecology and biochemistry. It is not the intention in this Section to discuss the extensive literature on the developmental physiology and biochemistry of seeds and factors controlling their dormancy and germination, in which investigators

have used excised and cultured embryos. Rather, the following discussion will deal specifically with the use of embryo culture method in some areas of seed biology in which practical applications have been developed or may be developed in the future.

4.1 Overcoming Seed Dormancy

Dormant seeds fail to germinate under apparently suitable conditions of adequate moisture and oxygen supply and optimum temperature unless they are given the appropriate dormancy-breaking treatment. Depending upon the treatments applied, a number of distinct forms of dormancy have been recognized (WAREING, 1969; VILLIERS, 1972). Since dormancy mechanisms in most cases hinge on the inability of the enclosed embryo to grow, special interest attaches to the role of embryo culture method in overcoming the developmental block.

Endogenous Inhibitors. Seeds of tall bearded *Iris* remain dormant for a period of time varying from a few months to many years after harvest. Recognition of the need for an effective method of breaking dormancy of seeds and obtaining flowers in a shorter period of time than normal led RANDOLPH (1945; RANDOLPH and COX, 1943) to study seedling production by embryo culture method. It was found that young seedlings with well-developed roots and leaves ready to be transplanted into soil could be obtained in about two or three months after transfer of embryos to a suitable medium. The use of embryo culture has done much to reduce the cycle from seed to flowering in *Iris* to less than a year, in contrast to the two or three years normally required for flowering, and its full potential is by no means reached. Later work with embryos of different species of *Iris* has established that seed dormancy is due to the presence of stable inhibitors of embryo growth present in the endosperm (RANDOLPH and COX, 1943; WERCKMEISTER, 1962), embryo (WERCKMEISTER, 1952; LENZ, 1955), or the seed coat (LENZ, 1955). Dormancy has been overcome in the seeds of other plants also by the embryo culture method, which has led to a recognition of the primary role of endogenous inhibitors in the process (COX *et al.*, 1945; MCLEAN, 1946; BULARD and DEGIVRY, 1965; DORE SWAMY and MOHAN RAM, 1967; LEPAGE, 1968; LEPAGE-DEGIVRY, 1970; LEPAGE-DEGIVRY and GARELLO, 1973; VAN STADEN *et al.*, 1972). The relative ease of extraction and culture of embryos from oil palm (*Elaeis guineensis*) seeds have made it possible to examine in detail a range of factors involved in dormancy, in particular, the age (RABÉCHAULT and AHEE, 1966), water content (RABÉCHAULT, 1967; RABÉCHAULT *et al.*, 1969) and the duration of soaking of seeds (RABÉCHAULT *et al.*, 1968).

Light. A large number of seeds exhibit a type of dormancy which is broken by light (positively photoblastic seeds), while in a limited number light inhibits germination (negatively photoblastic seeds). In the most widely investigated positively photoblastic seeds of lettuce (*Lactuca sativa*), it has been shown that while entire seeds remain incapable of germination even after prolonged periods in the dark, naked embryos freely grow in the dark. This has led to the suggestion that light exposure leads to the secretion of cytolytic enzymes which decompose

the endosperm and pericarp and overcome the mechanical resistance to embryo expansion (IKUMA and THIMANN, 1963). Supporting this view is the observation that dormancy can be reimposed in dark germinating isolated embryos by incubating them in an osmoticum such as mannitol (BEWLEY and FOUNTAIN, 1972). Embryo culture studies have shown that perhaps in a similar way, by controlling the mechanical restraint of the seed coats, or by interfering with gaseous exchange, light prevents the growth of the embryo and inhibits the germination of negatively photoblastic seeds of *Citrullus colocynthis* (KOLLER *et al.*, 1963), *Phacelia tenacetifolia* (CHEN and THIMANN, 1966; CHEN, 1970), and *Nemophila insignis* (CHEN, 1968).

Low Temperature. Seeds of a number of woody and herbaceous plants exhibit a type of dormancy that is overcome by prolonged exposure to low temperature (after-ripening). A stringent test for evaluating the effects of the chilling treatment is the ability of embryos excised from the after-ripened seeds to attain normal growth in culture. Such studies have revealed that the dormancy problem in low temperature-sensitive seeds is basically concerned with the physiological growth of the embryo, which appears morphologically fully formed. Generally, embryos excised from non-after-ripened seeds fail to grow in culture and full growth occurs only after the seeds or cultured embryos have been subjected to the appropriate duration of after-ripening treatment at the most favorable temperature. Embryo culture method has been extensively used to study the mechanism of the low temperature effect; such studies have provided valuable information on the endogenous inhibitors or promotors of growth in the embryos which are responsible for maintaining them in the dormant or nondormant state. For summaries of this interesting work, the reader is referred to the reviews of STOKES (1965), WAREING (1969) and VILLIERS (1972).

Dry Storage. Seeds of some of the common cereals are dormant immediately after harvest even though the embryo is fully mature. If the seeds are stored at dry temperatures, they gradually overcome dormancy and begin to germinate. In certain lots of seeds, which do not attain full germinability until after several years in storage, this type of dormancy may pose a major problem to the planter. Culture of embryos of dormant seeds of wild oat (*Avena fatua*) at different times after harvest has shown that the defect of completely isolated dormant embryos is overcome by addition of gibberellic acid to the medium (SIMPSON, 1965). Thus, culture of embryos of dormant grains in appropriate hormone-supplemented medium might be advantageously used to raise a new generation of seedlings from cereal grains immediately after harvest.

Immaturity of the Embryo. Orchid seeds are shed when the enclosed embryo is hardly developed beyond the globular stage, and, thus, they exhibit a form of dormancy imposed by the immaturity of the embryo. Morphological development of the embryo and its subsequent germination take place in the soil in association with a mycorrhizal fungus. KNUDSON (1922) succeeded in germinating orchid embryos into plantlets in the absence of the symbiotic fungus by growing them in a nutrient agar medium containing sugar. In the absence of sugar, embryos failed to develop beyond the protocorm stage. The asymbiotic

propagation of orchids, now practiced on an unparalleled commercial scale, has its inception in these experiments. Factors controlling the growth of orchid embryos in culture have been recently reviewed by ARDITTI (1967) to which source reference is made for further details.

4.2 Shortening the Breeding Cycle of Plants

In breeding practice, when dormancy of seeds and the slow growth of seedlings necessitate long breeding seasons, embryo culture method is of value to the grower in reducing the breeding cycle of new varieties. Cultivated varieties of rose generally take about a year to flower; and two to three months for the formation of fruits. Although excision of embryo from the seed is a rather tedious operation, seedlings originating from cultured embryos flower in two to three months. These flowers can serve as the male parent for further crosses, thus enabling the breeder to produce two generations in one year or shortening the breeding cycle to three or four months (LAMMERTS, 1946; ASEN, 1948). The value of embryo culture in circumventing the slow germination of the seed and the slow growth of the seedling is also illustrated in weeping crabapple (NICKELLS, 1951). In this plant, seedlings about four feet tall are obtained by embryo culture in about nine months, which is, incidentally, the time required for seeds to germinate in the soil.

There is also considerable information on the germination of seeds of some early ripening fruit trees which have very low viability and which fail to germinate even after appropriate after-ripening treatments. In general, it appears that embryos excised from such seeds grow normally in vitro into healthy plants (TUKEY, 1933, 1934, 1938; DAVIDSON, 1933, 1934). It is thus possible to preserve these varieties for development of still earlier ripening fruit characteristics which would be of practical application to the breeder. Often, excised embryos of progressively early ripening varieties show decreased growth potential in culture. Storage of fruits at a low temperature for a certain period can bring about normal growth of embryos excised from such fruits (LESLEY and BONNER, 1952; HESSE and KESTER, 1955; KESTER and HESSE, 1955a, b).

4.3 Overcoming Self-sterility of Seeds

The possibility of raising seedlings of crop plants which are traditionally propagated by vegetative means has been explored by embryo culture method. The classical example in this category of plants is the banana of which there are many seeded varieties. *Musa balbisiana* is a wild relative of the commercial banana, the seeds of which do not germinate in nature; however, if embryos are excised and grown in culture in a simple mineral salt medium, seedlings are readily obtained (COX *et al.*, 1960). With appropriate modifications this method could be applied to obtain seedlings from other varieties of banana. Similarly, the tuber crops, *Colocasia esculentum* and *C. antiquorum* are propagated only by vegetative means and seeds are never known to germinate in nature. The natural sterility barrier in the seed could be overcome by resorting to culture of embryos (ABRAHAM and RAMA-

CHANDRAN, 1960). Although these results are preliminary, the success of embryo culture method with these plants opens up new avenues for their improvements by interspecific and intervarietal hybridization.

4.4 Germination of Seeds of Obligate Parasites

It has proved difficult to germinate by conventional methods seeds of obligate phanerogamic root and stem parasites in the absence of contact with the host plant. In recent years, seeds and embryos of several obligate parasites have been grown under aseptic conditions to study their dependence on the host stimulus. Because some of the parasites prey upon economically important crops and trees causing ravage, an understanding of embryo morphogenesis will be informative.

Among the most widely studied obligate root parasites are members of Scrophulariaceae (for example, *Alectra*, *Buchera*, *Castilleja*, *Striga*), Orobanchaceae (*Cistanche*, *Orobanche*), and Santalaceae (*Santalum*). During seed germination, one end of the dimunitive embryo puts forth a germ-tube which superficially resembles a radicle. The tube penetrates the host root and by swelling and multiplication develops into a haustorium (WILLIAMS, 1958; RANGASWAMY, 1967). Several studies have shown that seed germination can be induced even in the absence of actual contact with the host plant by supplementing the medium with an exudate of the host root (OKONKWO, 1966), substances like kinetin, coumarin-type compounds (WORSHAM *et al.*, 1959, 1962), gibberellic acid (WILLIAMS, 1961), casein hydrolysate and coconut milk (RANGASWAMY and RAO, 1963; RANGAN, 1965; RANGASWAMY, 1967), or by exposure to light (OKONKWO and NWOKE, 1974). Thus, in general, the host appears to afford the seeds of the parasite specific substances of the type of growth hormones or amino acids which presumably trigger germination.

Obligate stem parasites belong mainly to Loranthaceae, Cuscutaceae, and Lauraceae. In contrast to the dimunitive embryos of root parasites, embryos of stem parasites are massive in size; furthermore, instead of a conventional root system, they have a haustorium at the radicular end which forms a graft with the host plant. Embryos excised from seeds of several stem parasites, notably, *Cuscuta reflexa* (MAHESHWARI and BALDEV, 1961), *Dendrophthoe falcata* (JOHRI and BAJAJ, 1962), *Amylotheca dictyophleba*, *Amyema pendula*, *A. miquelii* (JOHRI and BAJAJ, 1964), *Scurrula pulverulenta* (BHOJWANI, 1969), *Arceuthobium pusillum* (BONGA and CHAKRABORTY, 1968), *Cassytha filiformis* (RANGAN and RANGASWAMY, 1969), and *Phorandendron tomentosum* (BAJAJ, 1970) have been successfully grown in culture to the stage of plantlets. A common identifiable component of the medium necessary to induce growth in culture of embryos of many species was casein hydrolysate. Although auxins and other additives were occasionally employed along with casein hydrolysate, addition of IAA was decidedly inhibitory for growth of embryos of *Arceuthobium* (BONGA and CHAKRABORTY, 1968), and to some extent for embryos of *Cassytha* (RANGAN and RANGASWAMY, 1969).

While no effective treatments exist to control the damage caused to crop plants and trees by root and stem parasites, it is likely that knowledge of the mode of germination of their seeds and growth of embryos, and their sensitivity to

different additives in the medium, will offer particularly favorable opportunities to control seed germination and seedling growth on the host plant by chemical means.

4.5 Seed Testing

In seed testing trials, embryo culture method has figured as a rapid means of determining the viability of particular lots of seeds. TUKEY (1944) found a good correspondence between growth of excised embryos of non-after-ripened peach seeds and germination of after-ripened seeds. He has suggested culturing embryos from randomly selected mature non-after-ripened seeds as an index to determine the planting value of germination of seed lots several weeks earlier, while it might take months for completion of a normal germination test including the after-ripening treatment. From a practical point of view, nurserymen could use this as a quick test for predicting the viability of the current season's supply of seeds for specific planting dates, and thereby eliminate planting failures resulting from the use of seeds of low viability and expediate commercial movement of seeds of known germinability. In testing samples of corn of local origin in India, the growth of seedling from shriveled seeds was found to be similar under normal germination practice as well as by embryo culture method; this perhaps justifies the application of culture method to test the viability of samples of grains for seed purposes (MUKHERJI, 1951). By embryo culture method it has also been demonstrated that cold storage at freezing point will preserve the viability of young immature embryos of corn (UTTAMAN, 1949). A wider application of embryo excision method in seed testing practice has been predicated as a result of the success by which the degree of viability of seeds of several conifers, shrubs, vines, and fruit trees was accurately determined by this method (HEIT, 1955).

5. Other Applications

Although we have been concerned in the preceding Sections with the possible applications of embryo culture in horticulture and plant breeding, it should be kept in mind that the technique has been usefully employed to study some very fundamental problems in experimental embryogenesis. Thus, the progressive sophistication in culture methods and the increasing familiarity with their use have made it possible to study the growth requirements of progressively younger embryos, the effects of hormonal substances and environmental conditions on organogenesis in embryos, and the nutrition and metabolism of embryos during progressive embryogenesis. These studies have been reviewed extensively in recent years (NARAYANASWAMY and NORSTOG, 1964; MAHESHWARI and RANGASWAMY, 1965; WARDLAW, 1965; RAGHAVAN, 1966) and these reviews should be consulted to gain an overview of the contributions of plant embryo culture toward biological knowledge in general, and understanding plant development, in particular.

Table 1. Important plants in which embryo culture technique has been applied

Plant species	Purpose of embryo culture	Medium composition[a]	Reference
Abelmoschus esculentus × *A. manihot* hybrid	Overcome inviability	White's medium	PATIL (1966)
Abelmoschus esculentus × *A. moschatus*, *A. tuberculatus* × *A. moschatus* hybrids	Overcome inviability	Knop's major salts with Nitsch's minor elements and vitamins	GADWAL *et al.* (1968)
Avena fatua	Overcome seed dormancy	Modified Harris' medium with gibberellic acid	SIMPSON (1965)
Brassica pekinensis × *B. oleracea* hybrid	Overcome inviability	White's medium	NISHI *et al.* (1959)
Cattleya, *Laelia* and other orchids	Induce embryo growth in the absence of symbiotic fungus	Modified Pfeffer's medium	KNUDSON (1922)
Cerasus vulgaris × *C. tomentosa* hybrid	Overcome inviability	White's medium with yeast extract	KRAVTSOV and KAS'YANOVA (1968)
Chrysanthemum boreale × *C. pacificum* hybrid	Overcome inviability	White's medium	KANEKO (1957)
Colocasia esculentum, *C. antiquorum*	Overcome self-sterility of seeds	White's medium	ABRAHAM and RAMACHANDRAN (1960)
Corchorus capsularis × *C. olitorius* hybrid	Overcome inviability	White's medium with yeast extract, IAA and kinetin	ISLAM (1964)
Cucurbita pepo × *C. moschata* and reciprocal hybrids	Overcome inviability	Randolph's medium	WALL (1954)
Datura discolor × *D. stramonium* and other interspecific hybrids	Overcome inviability	Modified Tukey's medium with malt extract	SANDERS (1950)
D. innoxia × Tree Datura (*Brugmansia?*)	Overcome inviability	Tukey's or Randolph's medium with malt extract	BLAKESLEE and SATINA (1944)
D. stramonium × *D. ceratocaula* and other interspecific hybrids	Overcome inviability	Tukey's or Randolph and Cox's medium with malt extract	MCLEAN (1946)
Drosophyllum lusitanicum	Overcome natural seed dormancy	White's medium with or without casein hydrolysate	DORE SWAMY and MOHAN RAM (1967)
Elaeis guineensis	Overcome natural seed dormancy	White's medium with thiamine, calcium pantothenate and glutamic acid	RABÉCHAULT (1967)

Table 1. (continued)

Plant species	Purpose of embryo culture	Medium composition[a]	Reference
Gossypium davidsonii × *G. sturtii* hybrid	Overcome inviability	Dextrose-agar medium	SKOVSTED (1935)
G. hirsutum × *G. arboreum* and other interspecific hybrids	Overcome inviability	White's medium	BEASLEY (1940)
Hordeum sp. (barley)	Study effects of irradiated food materials on tissues	Irradiated potato mash	SWAMINATHAN *et al.* (1962)
Hordeum brachyantherum × *H. vulgare* and other interspecific hybrids; *H. californicum* × *Secale cereale* and other intergeneric hybrids	Overcome inviability	Randolph and Cox's medium	MORRISON *et al.* (1959)
H. jubatum × *Secale cereale* hybrid	Overcome inviability	White's medium with yeast extract	BRINK *et al.* (1944)
H. sativum × *H. bulbosum* hybrid	Overcome inviability	Randolph and Cox's medium	KONZAK *et al.* (1951)
H. vulgare × *H. bulbosum* hybrid	Overcome inviability	Randolph and Cox's medium	MORRISON *et al.* (1959); DAVIES (1960)
H. vulgare × (*H. compressum* × *H. pusillum*), *H. vulgare* × *H. hexopodium* hybrids	Overcome inviability	Mineral elements (magnesium, calcium, phosphorus, sulfur, potassium, nitrogen) and "Gibrel" (gibberellic acid)	SCHOOLER (1962)
Iris sp.	Accelerate seed germination	Randolph and Cox's medium	RANDOLPH and COX (1943)
Iris (tall bearded) × *I. tectorum* (crested *Iris*) hybrid	Overcome inviability	Nutrient agar medium	LENZ (1954)
Iris munzii × *I. sibirica* "Caesar's brother" hybrid	Overcome inviability	Nutrient agar medium	LENZ (1956)
Iris pallida I. macrantha, *I. pallida* × *I. chamaeris* hybrids	Overcome inviability	Pfeffer's medium	WERCKMEISTER (1936)
Iris pseudocorus × *I. versicolor* hybrid	Overcome inviability	Tukey's or Randolph's medium with malt extract	BLAKESLEE and SATINA (1944)
Lathyrus clymenum × *L. articulatus* hybrid	Overcome inviability	Inorganic medium of Bonner with thiamine and nicotinic acid	PECKET and SELIM (1965)
Lilium henryi × *L. regale* hybrid	Overcome inviability	Modified Knop's, Tukey's or White's medium	SKIRM (1942)

Table 1. (continued)

Plant species	Purpose of embryo culture	Medium composition[a]	Reference
Lilium speciosum "album" × *L. auratum*, *L. speciosum* "Rubrum" × *L. auratum* hybrids	Overcome inviability	Nutrient agar medium containing $Ca(NO)_3)_2$, $MgSO_4$, KH_2PO_4, KNO_3, $Fe_2(SO_4)_3$	EMSWELLER *et al.* (1962)
Linum perenne × *L. austriacum* hybrid	Overcome inviability	Damp blotting paper or cotton wadding containing glucose or sucrose	LAIBACH (1925)
Lotus corniculatus × *L. filicaulis* and other interspecific hybrids	Overcome inviability	Randolph and Cox's medium substituting chelated iron for ferrous sulfate	GRANT *et al.* (1962)
Lycopersicon esculentum × *L. peruvianum* hybrid	Overcome inviability	Hoagland and Snyder's, Tukey's or White's medium with coconut milk, malt extract, glycine, ascorbic acid, thiamine, riboflavin, nicotinic acid, pyridoxine, calcium pantothenate, succinic acid, and adenine as necessary	SMITH (1944); CHOUDHURY (1955); ALEXANDER (1956)
Malus sp. (Weeping Crabapple)	Accelerate seed germination	Medium containing mineral salts and vitamins	NICKELL (1951)
Medicago sativa and unnamed interspecific hybrids	Overcome embryo abortion due to self-sterility and interspecific inviability	Crone's nutrient solution	FRIDRIKSSON and BOLTON (1963)
Melilotus officinalis × *M. alba* hybrid	Overcome inviability	Crone's or Randolph and Cox's medium	WEBSTER (1955); SCHLOSSER-SZIGAT (1962)
Musa balbisiana	Overcome self-sterility of seeds	Knudson's or Randolph and Cox's medium	COX *et al.* (1960)
Oryza paraguaiensis × *O. brachyantha* and other interspecific hybrids	Overcome inviability	White's medium with coconut milk	LI *et al.* (1961)
Oryza sativa × *O. minuta*, *O. sativa* × *O.* sp. (Paraguay) hybrids	Overcome inviability	White's medium	NAKAJIMA and MORISHIMA (1958)

Table 1. (continued)

Plant species	Purpose of embryo culture	Medium composition[a]	Reference
Oryza sativa × *O. officinalis* and other interspecific hybrids	Overcome inviability	White's medium with malt extract and 10% coconut milk or Nitsch's medium with 10% coconut milk	IYER and GOVILA (1964)
Oryza sativa × *O. schweinfurthiana* and other interspecific	Overcome inviability	Mineral salt medium containing $Ca(NO_3)_2$,KNO_3, KCl, $MgSO_4$, $CaH(PO_4)_2$ and $FeSO_4$	BOUHARMONT (1961)
Phaseolus vulgaris × *P. acutifolius* hybrid	Overcome inviability	White's, Randolph and Cox's or modified Crone's medium	HONMA (1955)
Phaseolus vulgaris	Study host-parasite relationship in phytopathology	Nitsch's medium with vitamins	PADMANABHAN (1967)
Pinus lambertiana × *P. armandi*, *P. lambertiana* × *P. koraiensis* hybrids	Facilitate germination and overcome hybrid inviability	Plain agar slant	STONE and DUFFIELD (1950)
Prunus avium (sweet cherry)	Overcome low seed viability	Knop's medium or Crone's nitrogen-free medium	TUKEY (1933)
Prunus cerasus (sour cherry), *Prunus persica* (plum), *Pyrus communis* (pear), *Malus domestica* (apple) (intervarietal hybrids)	Overcome low seed viability	Robbins', White's, Crone's or Knop's medium	TUKEY (1934)
Prunus persica (plum)	Predict seed viability at planting	Damp peat moss, moist paper towel, or agar medium	TUKEY (1944)
Ribes nigrum × *Grossularia reclinata* hybrid	Overcome inviability	White's medium with yeast extract	KRAVTSOV and KAS'YANOVA (1968)
Rosa sp. (Rose)	Accelerate seed germination	Tukey's medium	ASEN (1948)
Solanum nigrum × *S. luteum* hybrid	Overcome inviability	Knop's solution	JØRGENSEN (1928)
Trifolium ambiguum × *T. hybridum* hybrid	Overcome inviability	Randolph and Cox's medium	KEIM (1953)
Trifolium repens × *T. nigrescens* and other interspecific hybrids	Overcome inviability	Tukey's or Randolph's medium	EVANS (1962)

Table 1. (continued)

Plant species	Purpose of embryo culture	Medium composition[a]	Reference
Tripasacum dactyloides × *Zea mays* hybrid	Overcome inviability	Randolph and Cox's medium	FARQUHARSON (1957)
Triticum durum × *Elymus arenarius* and other intergeneric hybrids	Overcome inviability	White's medium	IVANOVSKAYA (1946, 1962)
Triticum durum abyssinicum × *Secale cereale* hybrid	Overcome inviability	Mineral salts (NH_4NO_3, $MgSO_4$ $CaH_2(PO_4)_2$, KH_2PO_4, K_2HPO_4, Iron citrate) and casein hydrolysate	RÉDEI (1955)
Zea mays	Test seed quality	Modified White's medium	MUKHERJI (1951)

[a] In virtually all cases the media contained, in addition to the mineral salts, a carbon energy source such as glucose or sucrose. The varied types of mineral nutrients used by different investigators do not make it possible to give their composition here; references cited are recommended for further details.

In other studies, the use of embryo culture offers promise of extended application in studying host-pathogen interaction, as, for example, in the formation of ergot by infection of rye embryos by *Claviceps purpurea* (TONOLO, 1961) and *Fusarium* wilt of seedlings (PADMANABHAN, 1967). In the latter case, incorporation of the fungal toxin, fusaric acid, into the culture medium has been shown to interfere with the water uptake by germinated embryos of *Phaseolus vulgaris* and induce characteristic wilting of embryonic leaves. This observation is of great interest because sensitivity of embryos to the toxin would permit the study of its role in the plant using a system having adult organs in the form of primordial structures.

Cultured embryos have been used as test objects to evaluate the mutagenic ability of irradiated substrates on living tissues. When embryos of certain cereals were planted on X-irradiated nutrient medium and potato mash, or potato mash prepared from tubers which were irradiated with γ-rays and stored for prolonged periods, cytological abnormalities in abundance were observed in the embryonic cells, casting doubt on the wisdom of using irradiation procedures in food preservation (NATARAJAN and SWAMINATHAN, 1958; SWAMINATHAN *et al.*, 1962; CHOPRA and SWAMINATHAN, 1963).

Based on the discussion presented in this and the preceding Section, an alphabetical list of important genera and species of plants, in which embryo culture method has been successfully applied to overcome the disturbances in normal growth and reproductive processes, is given in Table 1.

6. Conclusions

The survey presented in this article should not be considered as anything more than a focus on the possible ways by which embryo culture method may be applied to alleviate some of the commonly encountered disturbances in the life of plants. Admittedly, this review is conspicuous for the lack of information dealing specifically with the use of embryo culture in field trials. As the general area of plant tissue culture enters a new era of possible commercial propagation of plants by asexual means (MURASHIGE, 1974), it is to be hoped that some attention will be paid to exploiting the potential of embryo culture method, where applications can be developed with little additional research.

References see page 442.

4. Triploid Plants through Endosperm Culture

B. M. Johri and S. S. Bhojwani

1. Introduction

The endosperm is a unique tissue. Being the fusion product of three haploid nuclei in most of the angiosperms (over 81% families), it is triploid. The endosperm is the main nutritive tissue for the embryo and also a dynamic centre of developmental influences on the embryo. Disfunction of endosperm, or its absence altogether, usually causes abortion of the embryo. The endosperm may be consumed wholly by the developing embryo so that the mature seed is nonendospermous, or it may persist and store abundant reserve food in the form of starch, fat, or proteins. During seed germination these substances are digested and utilized for the growth of the seedling until the latter develops the green pigments, and is able to synthesise its own food.

In angiosperms, the endosperm is a homogeneous mass of parenchymatous tissue, lacking the differentiation of vascular elements. While all other ovular tissues have been reported to form embryos in nature, there is no convincing demonstration of the endosperm doing so, even as an abnormality. Being devoid of differentiation, the endosperm offers an excellent system for experimental morphogenic studies.

During the past decade the potentiality of endosperm tissue for organogenic differentiation in vitro has been well established. Since the endosperm tissue is mostly triploid, the plantlets formed from them are also triploid. The significance of triploids in plant improvement is well-known. They may be exploited for the seedless nature of their fruits, especially when numerous seeds are embedded in the pulp, e.g. watermelon. Triploids of some plants are superior to their diploids and tetraploids with reference to the commercial product/s. They can also be utilized to obtain trisomic lines for genetic mapping. The technique of endosperm culture may offer the plant breeders a new method for raising triploids.

In this article we shall first deal with growth and differentiation in tissue cultures of endosperm and then discuss the possibility of using the technique of endosperm culture for raising triploids in relation to plant improvement.

2. Earlier Studies on Endosperm Culture

To the best of our information the earliest attempt to grow endosperm tissue in cultures was that by Lampe and Mills (1933; cited by LaRue, 1936). They grew young corn *(Zea mays)* endosperm on a nutrient medium (containing the extract

of potato or young corn), and obtained slight proliferation of cells adjacent to the embryo. However, the first extensive work on growth and differentiation of endosperm tissue in cultures was undertaken by LARUE and his associates at the University of Michigan, Ann Arbor, USA. After several years of sustained work, in 1949 LARUE, for the first time, reported the establishment of a continuously growing tissue from immature maize endosperm. Subsequently, many other workers examined tissue cultures of maize from diverse angles. During 1949 and 1962 tissue cultures were established from immature endosperm of *Asimina triloba* (LAMPTON, 1952), *Cucumis sativus* (NAKAJIMA, 1962), and *Lolium perenne* (NORSTOG, 1956). A common feature of all these reports was that only immature endosperm of the right age could be grown in cultures. The ability of cells from mature endosperm to divide was first demonstrated by MOHAN RAM and SATSANGI in 1963. Two years later these authors announced the establishment of tissue cultures from mature endosperm of *Ricinus communis* (SATSANGI and MOHAN RAM, 1965). In his early papers LARUE refers to organogenic differentiation in endosperm cultures of castor bean (1944) and maize (1947), but neither of these reports could be confirmed by subsequent workers who undertook extensive works on these two systems.

3. Callusing from Endosperm Tissue

To date both immature and mature endosperm have been successfully grown in cultures (Table 1). The cereal endosperm proliferates only if excised during a proper period of development: 9–10 days after pollination (DAP) in *Lolium*, and 8–11 in corn. The mature endosperm is not amenable to culture. TAMAOKI and ULLSTRUP (1958) have suggested that certain physiological changes occur in corn endosperm 12 DAP that render it incapable of responding to the treatments in cultures. The plant physiologists regard the mature endosperm of cereals as dead, except for a few outer layers which constitute the aleurone tissue (VARNER, 1971). Interestingly, a dicot (*Cucumis sativus*) has also been reported to exhibit similar behavior (NAKAJIMA, 1962); the endosperm proliferates only if it is excised 7–10 DAP.

During the last decade continuously growing tissues have been established from the mature endosperm of several dicots. The plants which have so far responded favourably belong to the families Euphorbiaceae, Loranthaceae, and Santalaceae (JOHRI and SRIVASTAVA, 1973a). With respect to the first two families, it was observed that the initial association of the embryo was essential for inducing proliferation. Shortly after the endosperm had started callusing, the embryo could be removed without affecting the growth of the tissue. When mature endosperm with the embryo intact is planted on a proper nutrient medium, the embryo shows the initial stages of germination, such as elongation of hypocotyl, and expansion of cotyledons (BHOJWANI and JOHRI, 1971). If not removed at this stage, the embryo may also proliferate and mixed embryo-endosperm callus would be formed. It is, therefore, advisable to remove the embryo soon after the endosperm cells have started dividing.

Table 1. Chief morphogenic responses of cultured endosperm

Taxa	Explant	Media	Response	Reference
Zea mays Gramineae	Immature endosperm	BM + YE	Tissue cultures established for the first time	LaRue (1949)
	Endosperm 12 DAP	BM + Asparagine	Growth of callus is as good as on BM + YE	Straus and LaRue (1954)
Asimina triloba Annonaceae			Tissue cultures established	Lampton (1952)
Lolium perenne Gramineae	Endosperm 9–10 DAP	BM + YE	Tissue cultures established	Norstog (1956)
Cucumis sativus Cucurbitaceae	Endosperm 10 DAP	BM + IAA + DPU + CH	Tissue cultures established	Nakajima (1962)
Santalum album Santalaceae	Dried "seeds"	BM + 2,4-D + KN + YE	Tissue cultures from mature endosperm raised for the first time; mature endosperm did not proliferate in the absence of embryo	Rangaswamy and Rao (1963)
Ricinus communis Euphorbiaceae	De-coated dried seeds	BM + 2,4-D + KN + YE	Tissue cultures of endosperm established; callus differentiated tracheidal cells and embryo-like structures	Satsangi and Mohan Ram (1965)
	Endosperm from germinated seeds	BM + 2,4-D + KN + YE	Excised mature endosperm from germinated seeds formed callus; endosperm from dried seeds failed to grow	Brown *et al.* (1970)
Exocarpus cupressiformis Santalaceae	Dried "seeds"	BM + IAA + KN + CH	Numerous, triploid shoot buds differentiated directly from the endosperm	Johri and Bhojwani (1965)
Croton bonplandianum Euphorbiaceae	De-coated dried seeds	BM + 2,4-D + KN + YE	Numerous tracheidal cells and embryo-like cell aggregates differentiated from endosperm callus	Bhojwani (1966)
	Endosperm callus subcultured	BM or BM + CH	Roots differentiated from callus	Bhojwani and Johri (1971)
Scurrula pulverulenta Loranthaceae	Mature "seeds"	BM + cytokinin	Shoot buds differentiated from peripheral cells of endosperm	Bhojwani and Johri (1970)
	Buds from endosperm	BM + IAA + KN + CH	Buds callused followed by differentiation of buds and haustoria	

Table 1. (continued)

Taxa	Explant	Media	Response	Reference
Phoradendron tomentosum Loranthaceae	De-coated mature seed	BM + 2,4-D + KN + YE	Endosperm proliferated to form callus; differentiation of papillae-like outgrowths	BAJAJ (1970)
Dendrophthoe falcata Loranthaceae	Mature "seeds"	BM + IAA + KN + CH	Endosperm proliferated, and differentiated; shoots and haustoria; increase in IAA promoted haustorium formation and increase in KN enhanced shoot bud formation	BAJAJ (1968); NAG and JOHRI (1971)
Taxillus vestitus Loranthaceae	Mature "seeds" or excised endosperm	BM + cytokinin	Shoots developed from endosperm; presence of embryo suppressed bud differentiation; in the cultures of endosperm pieces buds first developed from epidermal cells adjacent to cut surface in contact with the medium	NAG and JOHRI (1971)
Jatropha panduraefolia Euphorbiaceae	De-coated mature seed	BM + 2,4-D + KN + YE	Profusely growing endosperm callus differentiated tracheidal cells	SRIVASTAVA (1971)
	Endosperm callus	BM + NAA + KN + CH	Frequent differentiation of shoots and roots; elimination of NAA supported only shoot formation whereas elimination of KN favoured only root formation	
Putranjiva roxburghii Euphorbiaceae	De-coated mature seeds	BM + IAA + KN + CH	Profusely growing endosperm callus formed; after 4 weeks roots and shoots differentiated from callus and plantlets formed	SRIVASTAVA (1973)
Oryza sativa Gramineae	Endosperm 4–7 DAP	BM + 2,4-D + YE	Tissue cultures established; elimination of 2,4-D brought about the differentiation of many roots and a couple of plantlets	NAKANO *et al.* (1975)

Abbreviations: *BM*: Basal medium *YE*: Yeast extract *CH*: Casein hydrolysate *KN*: Kinetin *DPU*: 1,3-diphenylurea, *DAP*: Days after pollination, *De-coated*: Seed coat removed.

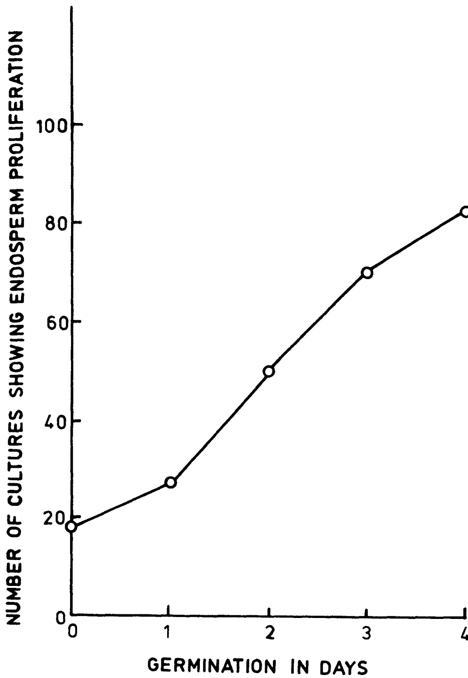


Fig. 1. *Ricinus communis:* effect of germination-period on the proliferation of excised endosperm; zero day refers to seed soaked for 22 h. (Drawn from the data BROWN *et al.*, 1970)

The role of embryo in the proliferation of mature endosperm is at present a matter of speculation. It is evident from the work of BROWN *et al.* (1970) that some factor or factors are contributed by the germinating embryo which are essential for the activation of mature and dried endosperm of castor bean (Fig. 1). These workers observed that endosperm pieces from dry seeds of castor bean did not grow in cultures. However, if endosperm was excised from seeds soaked in 3.5% $Ca(OCl)_2$ solution for 22 h, some explants did proliferate. The number of proliferating endosperm pieces further increased if the soaked seeds were allowed to germinate, and there was a direct relationship between the number of days after seed germination and the number of cultures showing endosperm proliferation (Fig. 1). It has been demonstrated that during germination the embryo releases gibberellin-like substances (OGAWA, 1964; INGLE and HAGEMAN, 1965). In cereal endosperm gibberellin is known to help in the activation and de novo synthesis of certain enzymes. BHOJWANI (unpublished) also noted that GA_3 was able to replace the "embryo factor" for inducing proliferation of mature endosperm of *Croton bonplandianum.*

3.1 Growth Factors

LARUE (1949) cultured corn endosperm on media containing various supplements such as tomato juice (TJ), grape juice, green corn juice, yeast extract (YE), or cow's milk. Of all these, 20% TJ supported maximal growth. STERNHEIMER (1954) confirmed the superiority of TJ for corn endosperm callus. However, STRAUS and

LARUE (1954) observed that the callus growth on TJ-supplemented medium was erratic and unpredictable. These investigators used canned TJ and, therefore, it is unlikely that every time the juice which was added to the medium came from tomatoes of the same age. Cytokinin-like activity in TJ is known to decrease correspondingly with the age of the fruit (BOTTOMLEY *et al.*, 1963). This may be one of the reasons for the erratic and unpredictable growth of corn endosperm when TJ was added in the nutrient medium.

STRAUS and LARUE (1954), and TAMAOKI and ULLSTRUP (1958) demonstrated that YE could substitute for TJ to a considerable extent. Yeast extract also supported good growth of *Lolium* endosperm callus (NORSTOG, 1956). In the process of developing a purely synthetic medium, STRAUS (1960) found asparagine (1.5×10^{-2}) to be superior to TJ or YE. NAKAJIMA (1962) carried out a series of experiments, and reported that for satisfactory growth of endosperm callus of *Cucumis* a combination of an auxin (IAA), a cell division factor (1,3-diphenylurea), and a source of organic nitrogen (casein hydrolysate) was necessary. Later, it was shown that mature endosperm tissue of *Santalum* (RANGASWAMY and RAO, 1963), *Ricinus* (SATSANGI and MOHAN RAM, 1965), *Croton* (BHOJWANI and JOHRI, 1971), and *Jatropha* (SRIVASTAVA, 1971) grow best on a nutrient medium supplemented with 2,4-D, kinetin, and YE.

Unfortunately, not many workers have paid attention to the influence of physical factors—such as light, temperature, and pH—on the growth of endosperm tissue. The corn endosperm grows better when maintained in the dark (STRAUS and LARUE, 1954), whereas *Ricinus* endosperm grows best under continuous light (JOHRI and SRIVASTAVA, 1973a). The optimum temperature for endosperm callus is reported to be around 25 C. The optimum pH of the culture medium varies from 4.0 for *Asimina* (LAMPTON, 1952) to 5.0 for *Ricinus* (JOHRI and SRIVASTAVA, 1973a) and 6.1 for corn (STRAUS and LARUE, 1954).

3.2 Histology and Cytology

In *Zea mays* the endosperm 12 days after pollination comprises a comparatively homogeneous tissue with a single peripheral layer of meristematic cells. In cultures the cells of this layer undergo anticlinal and periclinal divisions to add to the girth of the outermost layer as well as to the mass of general endosperm tissue. By the third day the peripheral layer becomes 4 cells thick and, during the next 24 h it adds up many more layers. This is followed by differential growth at localised areas leading to the formation of nodules (Fig. 2C). These meristematic nodules may arise directly from the peripheral layer, or cells located immediately below the outermost layer (STRAUS, 1954; SEHGAL, 1969).

On a medium fortified with 2,4-D, kinetin, and yeast extract the proliferation of mature endosperm begins 10–12 days after inoculation. In *Croton* the callusing starts on the inner side of the endosperm and becomes visible when the endosperm bursts open into two halves (Fig. 2A). The embryo is removed at this stage, and the proliferation extends all over the endosperm tissue (Fig. 2B). On the same medium *Santalum* endosperm starts callusing with the differentiation of concentric layers of meristematic tissue about 5–6 layers below the epidermis (RANGAS-

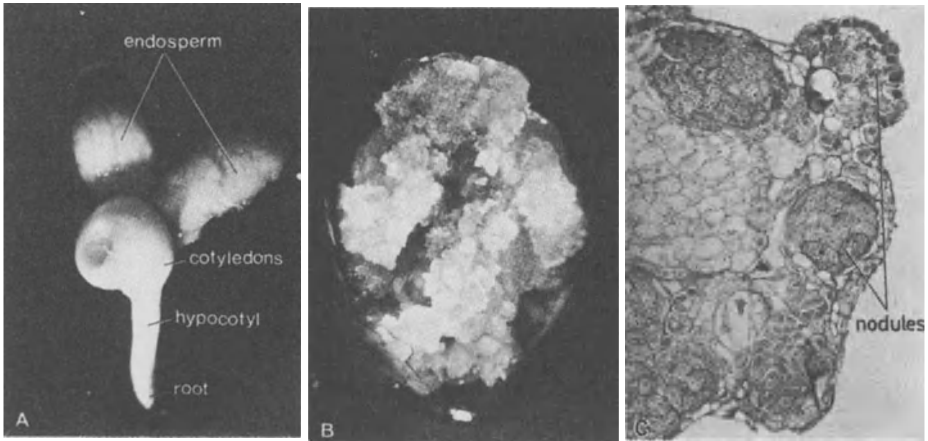


Fig. 2. (A, B) *Croton bonplandianum*; C. *Zea mays*: in vitro proliferation of endosperm. (A) 10-day-old culture of de-coated seed showing germinated embryo with enlarged and coiled cotyledons, and elongated hypocotyl; also note proliferation on the inner side of two halves of endosperm. (B) Profusely growing endosperm callus. (C) Section of a 4-week-old culture; note hypodermal nodules. (A, B) After BHOJWANI and JOHRI, 1971; (C) after SEHGAL, 1969)

WAMY and RAO, 1963). In the presence of kinetin alone, or in combination with IAA, the callusing is initiated with the differentiation of localised, peripheral meristems leading to the formation of nodular outgrowths on the surface of endosperm.

Histological studies of the cultured endosperm at the time of callus initiation have revealed that the cells of the meristematic zone are always free from reserve food material, whereas the quiescent cells of the endosperm are rich in it (NAG and JOHRI, 1971). With the passage of time in culture most of the callus cells become depleted of their reserve-food contents.

Irrespective of the composition of culture medium, the endosperm callus of cereals remains purely parenchymatous. This is also true for *Cucumis* (NAKAJIMA, 1962) and *Santalum* (RANGASWAMY and RAO, 1963). However, tissues derived from the endosperm of euphorbiaceous, loranthaceous, and most of the santalaceous members readily differentiate tracheidal elements (JOHRI and SRIVASTAVA, 1973b). The tracheidal elements may appear scattered or in clusters. The slow-growing and compact calli show a higher degree of tracheidal differentiation. Organogenic differentiation from endosperm tissue occurs only in those plants which exhibit tracheidal differentiation in cultures.

The endosperm tissue is well-known for a high degree of polyploidization of its cells during in vivo development. It also exhibits various kinds of mitotic irregularities such as chromosome bridges and lagging chromosomes. These features are also common in long-term tissue cultures of various organs including endosperm. STRAUS (1954) reported that in established cultures of corn endosperm, the polyploid, hypoploid, and aneuploid cells were as common as those showing normal chromosome number. Thirty percent of the total cells observed in anaphase exhibited chromosome bridges and lagging chromosomes. Cells of

ploidy higher than 3n have also been reported in endosperm cultures of *Croton*, *Jatropha*, and *Lolium*. It is, however, interesting that in an over 10-year-old callus of rye-grass endosperm majority of the cells remained triploid (NORSTOG *et al.*, 1969). A remarkable stability in chromosome number of endosperm cells in vivo as well as in vitro is exhibited by *Dendrophthoe falcata* (JOHRI and NAG, 1974). The composition of the nutrient medium (TORREY, 1965), and position of the explants on the medium (MATTHYSSE and TORREY, 1967) are known to influence polyploid mitoses in the pea. Neither of these factors or treatments could alter the chromosomal constitution of endosperm cells in *Dendrophthoe*. Probably, in this taxon the natural mechanism leading to polyploidy is either lacking or inoperative, as described for *Helianthus* by PARTANEN (1965).

4. Organogenesis

As early as 1944, LARUE reported that excised endosperm pieces of castor bean regenerated roots. Again, in 1947 the same author published that in corn endosperm cultures "... less than one in a thousand developed roots and a single one formed root-shoot axis with miniature leaves". However, neither of these communications described the nutrient medium or physical conditions which favoured differentiation. Moreover, in their detailed studies on endosperm cultures of *Ricinus*, SATSANGI and MOHAN RAM (1965), and JOHRI and SRIVASTAVA (1973a) never obtained organogenic differentiation. Regarding the corn endosperm, STRAUS (1954), one of LARUE's students, remarked: "Since then, the tissue has passed through approximately 95 transfers and has produced an estimated 15 kg of tissue. Not a single example of complex differentiation was observed during this period."

The first convincing demonstration of organ formation from cultured endosperm tissue is in *Exocarpus cupressiformis* by JOHRI and BHOJWANI (1965). They observed that in 'seed' (in Loranthaceae and Santalaceae the seed lacks the seedcoat) cultures of *Exocarpus* on a medium supplemented with IAA, kinetin, and casein hydrolysate (CH), 10% cultures formed shoot buds all over the endosperm. In a single explant as many as eight buds developed. The origin of these buds from endosperm was confirmed through histological studies. In situ the buds did not grow beyond 1.25 cm. On the other hand, if the buds were excised and planted on a fresh medium of the same composition, they formed a slow-growing callus which later differentiated shoot buds. These buds also showed limited growth. The buds were, as expected, triploid.

Stimulated by the report of JOHRI and BHOJWANI, extensive studies were undertaken to raise full triploid plants through endosperm culture. To date, organogenic differentiation in endosperm cultures has been achieved in *Dendrophthoe falcata*, *Leptomeria acida*, *Phoradendron tomentosum*, *Scurrula pulverulenta*, *Taxillus vestitus*, *T. cuneatus*, *Croton bonplandianum*, *Jatropha panduraefolia*, *Oryza sativa* and *Putranjiva roxburghii* (Table 1). Of these the first six taxa are semi-parasites, while the other three are autotrophs.

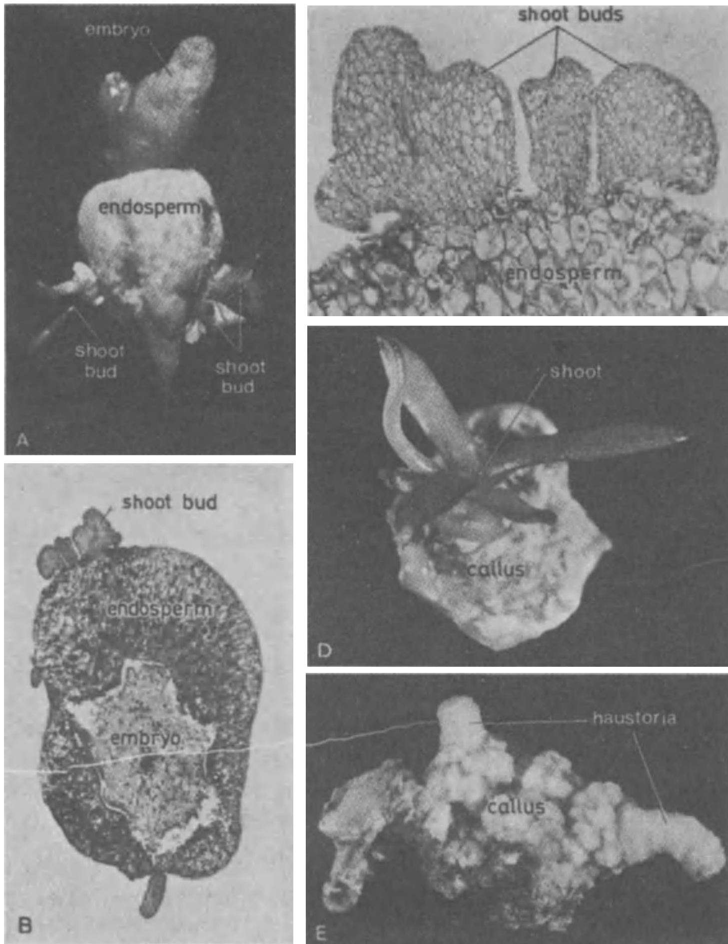


Fig. 3A–E. ***Scurrulla pulverulenta:*** **(A) 16-week-old culture of "seed" on White's medium supplemented with zeatin (10^{-5}M).** Note the differentation of buds from endosperm. (B) Transection of cultured "seed" showing buds arising from the periphery of endosperm. (C) Portion from (B) to demonstrate the peripheral origin of buds. (B, E) Endosperm-buds subcultured on a medium enriched with IAA, Kinetin, and CH. The callused buds have formed shoot (D), and haustorium (E). (After BHOJWANI and JOHRI, 1970)

The endosperm may form buds either directly, or it may first proliferate into a callus mass followed by organogenesis. For shoot bud differentiation from endosperm tissue an exogenous cytokinin is always necessary. In "seed" cultures of *S. pulverulenta*, BHOJWANI and JOHRI (1970) observed that on a medium supplemented with IAA, kinetin, and CH, 15% of the cultures showed surface callusing of the endosperm. In another 13% green buds appeared from the unproliferated regions of endosperm (Fig. 3A). It was subsequently shown that if CH was omitted from the above medium, the percentage of cultures showing bud differentiation rose to 26. The studies on the role of IAA and kinetin revealed that kinetin alone was more effective than in combination with IAA; there was no differentiation in

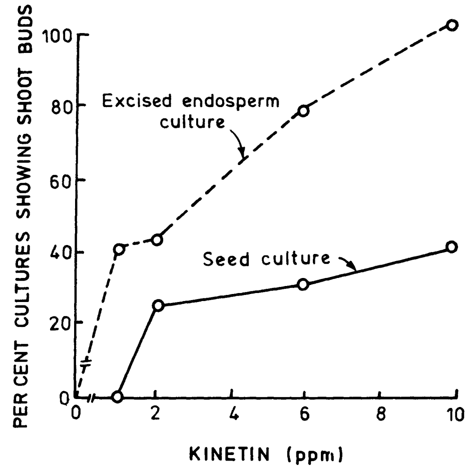


Fig. 4. *Taxillus vestitus:* influence of kinetin and the presence of embryo on shoot bud differentiation from endosperm. (After JOHRI and NAG, 1970)

the absence of kinetin, and IAA counteracted the effect of kinetin. Furthermore, with increase in the concentration of kinetin from 1 mg/l to 10 mg/l, there was a corresponding increase in the number of cultures forming buds from endosperm. *Taxillus vestitus* endosperm shows a similar response (Fig. 4). However, in *Dendrophthoe* and *Leptomeria*, kinetin-induced bud differentiation only in the presence of a low concentration of auxin, such as IAA or IBA. Here also an increase in the concentration of kinetin enhanced the response, whereas increase in the concentration of auxin antagonised it (NAG and JOHRI, 1971). Endosperm pieces of *Taxillus vestitus*, presoaked in 0.025% kinetin solution, for 24 h, differentiated buds even on White's basal medium (lacking kinetin). Of the various cytokinins so far tested for bud formation from endosperm, 6-(γ,γ-dimethylallylamino)-purine proved most effective, and triacanthine the least (Fig. 5; BHOJWANI and JOHRI, 1970; JOHRI and NAG, 1970).

In all other systems where cytokinins have been reported to favour bud formation, it is a case of promotion rather than induction. In this connection MILLER (1961) remarked that the fact to be kept in mind is that all of the plants reported to respond to kinetin in terms of increased budding show some formation of shoot structures, even in the absence of kinetin. Thus, kinetin is known to cause bud formation only in those plant materials with an inherent tendency for such formation. The endosperm tissue neither forms buds in vivo nor in cultures in the absence of a cytokinin. It is, therefore, a distinct case of bud induction by cytokinins.

In *Taxillus*, the embryo has an adverse effect on bud differentiation from endosperm. The number of buds per culture, and number of cultures forming buds (Fig. 4), is comparatively higher when endosperm halves are cultured, as compared to that when endosperm with the embryo intact is cultured. However, subsequent development of buds is better in the presence of the embryo. Injury on the endosperm enhances the response (Fig. 6). Also, the position of the endosperm on the culture medium has a significant effect on the differentiation and distribution of buds (Fig. 6). Irrespective of the position of endosperm on the medium, the buds invariably first appear along the injury (Fig. 6 BB′, EE′).

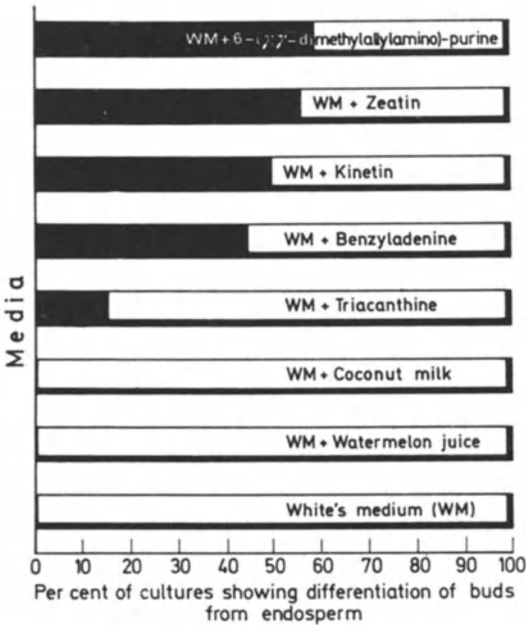


Fig. 5. *Scurrula pulverulenta*: influence of various cytokinins, coconut milk, and watermelon juice on shoot bud formation from endosperm. *Solid portion of bars*: percentage cultures forming buds. (After BHOJWANI and JOHRI, 1970)

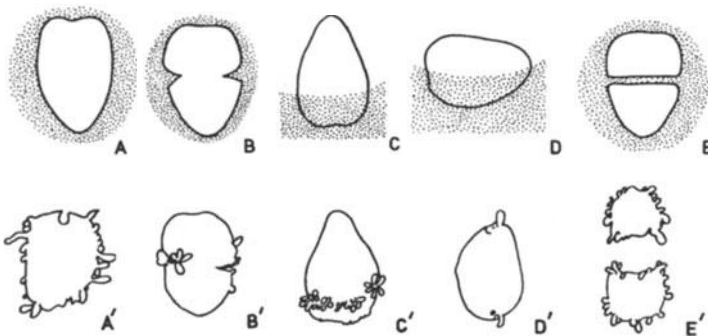


Fig. 6 A–E. *Taxillus vestitus*: effects of injury, and the position of explant on semi-solid medium, on bud differentiation from endosperm. *Stippled portion*: the medium. (A–E) Treatment, (A'–E') responses. (A, A') Half-split (longitudinally) endosperm with cut surface in contact with medium. (B, B') Two vertical pieces removed from endosperm, and planted as in (A). (C, C') Same as (C) but planted horizontally. (E, E') Half-split endosperm cut into two transverse pieces, and planted as in (A). Invariably, the buds arise close to the injury; just above the level of medium. (After NAG and JOHRI, 1971)

If half-split endosperm of *Taxillus vestitus* is planted with the cut surface in contact with the medium (White's medium with kinetin 5 mg/l), 100% cultures form 12–18 buds (Fig. 6AA'), whereas if the cut surface is kept away from the medium, only 1–3 buds develop in 30% cultures. The site of bud formation (in these segments) was almost predictable. If the half-split endosperm was planted vertically or horizontally the buds first appeared from the epidermal cells adjacent the cut end situated immediately above the level of medium (Fig. 6CC', DD').

The buds formed directly from the endosperm (without callusing) show limited growth in situ. If these buds are excised and planted on a fresh medium of the same composition, the buds, instead of growing into larger shoots, proliferate into a compact callus. These calli, as well as those derived by direct proliferation of the endosperm tissue, differentiate shoot buds and/or haustoria (Fig. 3D, E). In *Dendrophthoe*, *Nuytsia*, and *Taxillus* a tissue of unlimited growth was obtained on White's medium fortified with IBA 5 mg/l, kinetin 5 mg/l, and casein hydrolysate 2000 mg/l. If IBA (in the above combination) was replaced by IAA 2.5 mg/l, the endosperm callus in *Dendrophthoe* and *Taxillus* differentiated buds and haustoria. An increased concentration of kinetin enhanced bud formation and suppressed haustoria formation, whereas an increase in the concentration of IAA promoted haustoria formation and adversely affected shoot formation. Being stem parasites, *Dendrophthoe*, *Leptomeria*, *Scurrula*, and *Taxillus* lack a true root system. Instead, haustorial structures are formed.

The differentiation of buds from intact endosperm in *Scurrula pulverulenta* occurs as a result of meristematic activity of the epidermal as well as hypodermal cells (Fig. 3B, C) which, as compared to the cells of deeper region of endosperm, are

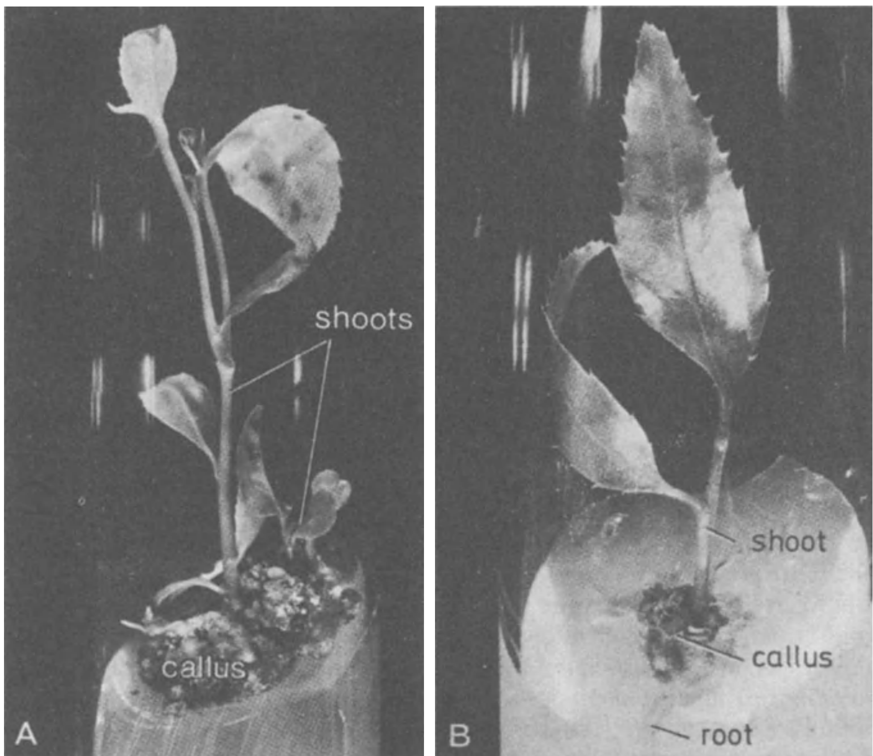


Fig. 7A and B. *Putranjiva roxburghii:* plantlet formation from endosperm callus. (A) Well-differentiated shoots with normal leaves. (B) Differentiation of root and shoot from callus. (After SRIVASTAVA, 1973)

smaller and richly cytoplasmic. In *Taxillus* the buds originated exclusively from the epidermal cells. The initiation of buds had no correlation with the differentiation of vascular tissue (Fig. 3B, C). The young buds appeared as mere parenchymatous outgrowths and, subsequently, vascular traces differentiated in the buds. The endosperm tissue per se, however, did not show any vascular differentiation.

The autotrophic taxa that have exhibited organogenesis in endosperm cultures are *Croton bonplandianum*, *Jatropha panduraefolia*, *Putranjiva roxburghii*, all belonging to the family Euphorbiaceae and *Oryza sativa* of Gramineae. In these plants differentiation of organs usually occurs from callused endosperm. The calli of *Croton* and *Jatropha* grow best on a medium supplemented with 2,4-D, kinetin, and YE but, on this medium the tissue remains unorganised (BHOJWANI, 1966). Upon transfer to the basal medium, the endosperm callus of *Croton* forms numerous roots which grow to various sizes (BHOJWANI and JOHRI, 1971). All attempts to induce bud formation from this tissue have so far proved futile. The endosperm calli of *Jatropha* and *Putranjiva* (SRIVASTAVA, 1971) (Fig. 7A, B) form roots as well as shoots. In the latter, root and shoot may differentiate as a bipolar axis. As mentioned earlier, the endosperm callus of *Putranjiva* grows best on a medium supplemented with IAA, kinetin, and CH. On the same medium, the callus assumes nodulated appearance followed by organogenesis. After 8 weeks about 80% of the cultures formed buds (SRIVASTAVA, 1973). Within the next 8 weeks the shoots attained a height of about 4 cm, and bore 3 or 4 pairs of leaves. In about 20% cultures the root-shoot axis was quite distinct. These plantlets of endosperm origin have yet to be successfully transplanted to soil.

It is interesting that the organs and plantlets differentiated from endosperm tissue are invariably triploid; morphologically and anatomically they are comparable to their counterparts formed by the zygotic embryo.

5. Importance of Triploids in Plant Improvement

The triploids are usually seed-sterile and, consequently, undesirable for plants where seeds are of commercial importance. However, there are some situations where seedlessness caused by triploidy is of no serious concern or, at times, even advantageous. It is with reference to these plants that triploidy can be exploited for plant improvement. Some of the economically important plants whose triploids are presently in commercial use include several varieties of apple, bananas, mulberry, sugar beets, tea, and watermelons (see ELLIOTT, 1958).

As compared to their diploids, the triploid quaking aspen (*Populus tremuloides*) has more desirable pulpwood characteristics of interest to forest industry. Seed-sterility in this plant is not a serious set-back because it can be easily multiplied vegetatively (WINTON, 1970). Similarly, for the crops grown for their vegetative parts, seedlessness of the triploids should not matter provided the plants are otherwise superior. One difficulty may, however, arise if the plants cannot be vegetatively propagated, and that is in the repeated production of triploid seeds. The most common method for triploid production is to cross the

tetraploids with diploids. In different situations this cross may not be successful and, therefore, the repeated production of triploid seeds would be difficult. When such a contingency arises, an alternative technique is to be adopted.

From the above account it is evident that triploid plants in large numbers can be raised through endosperm cultures. It is now time that plant breeders apply the techniques of endosperm culture to economically important plants for raising superior triploid plants where the conventional method of crossing tetraploids and diploids fails or proves difficult.

References see page 442.

5. Applications of in vitro Pollination and in vitro Fertilization

N. S. RANGASWAMY

1. Introduction

Pollination is the transference of pollen grains from the anther to the stigma. In vivo this is accomplished by various agencies. In the normal way various events ensue pollination: the pollen grains germinate on stigma; the pollen tubes grow through stigma, style, and placenta, and finally carry the immotile male gametes into ovules enclosed inside the ovary; the two male gametes are liberated inside the embryo sac; one male gamete fuses with the egg nucleus and the other with the secondary nucleus; and thus the fertilization is completed. Most angiosperms are outbreeders, and therefore they preclude self-pollination. Likewise, natural interspecific and intergeneric hybridizations are also rare. But in plant breeding programs selfing and hybridization are well-recognized methods of obtaining desirable results. Obviously any technique which would lead to achieving either selfing or hybridization, or both, is of practical importance. In vitro pollination and fertilization is one such technique.

This article describes the establishment of the technique of in vitro pollination and fertilization, the achievements already obtained through this technique in plant breeding and genetics, the factors which affect the technique, and its potential in applied and fundamental botany.

2. The Technique

Two steps are basic to the technique of in vitro pollination, fertilization, and seed development: (1) using pollen grains and ovules at the right stage, and (2) determining a nutrient medium or media which would support optimal pollen germination and pollen tube growth, and development of seeds to maturity. Primarily these involve a temporal study in vivo of the following aspects of floral biology of the experimental material: (1) anthesis, (2) dehiscence of anthers, (3) pollination, (4) pollen germination, (5) pollen tube growth and entry into ovules, (6) fertilization, and (7) seed maturation.

Only a brief account of the methods of collecting the pollen and ovules, and raising the cultures, is given here. Collection of pollen under aseptic conditions is best achieved by excising aseptically the ready-to-dehisce anthers from previously chosen flower buds; if anthers have to be excised from flowers, the excised anthers are surface-sterilized. In either case, the excised anthers are allowed to dehisce on a suitable substratum (such as filter paper) in presterilized

containers, e.g. petri dishes, and the pollen from the is used for in vitro pollination.

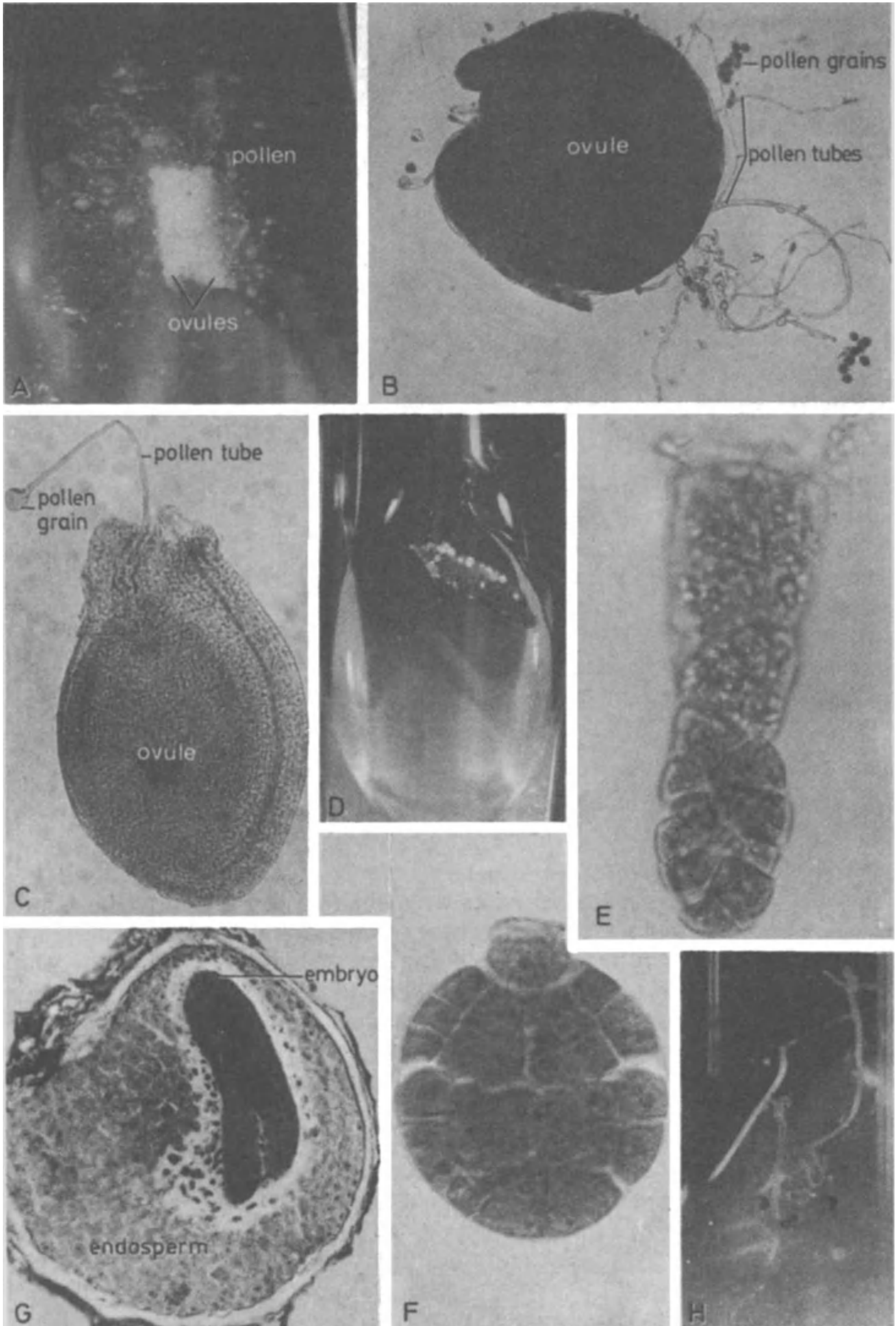
To prevent chance pollination in vivo, flower buds are emasculated (in bisexual species) and bagged prior to anthesis. To carry out stigmatic pollinations in pistil cultures, the emasculated and bagged flower buds are collected a day before or on the day of anthesis, and all the following operations are conducted inside a sterilized chamber. First, all parts of the flower bud except the pistil (and pedicel when present) are clipped off; as an additional precaution the pistil is surface-sterilized with a liquid agent without wetting the stigma; the pistil is carefully wiped dry; (the pedicel is suitably shortened); the stigma is pollinated with the pollen obtained under aseptic conditions as already described; finally the pollinated pistil is implanted in the sterilized agar nutrient medium. Pistils excised before anthesis are maintained in sterile cultures until they are pollinated under aseptic conditions, and are promptly returned to the culture vials. The cultures are grown under controlled conditions of light and temperature, and humidity if possible.

For carrying out placental and ovule pollinations in vitro, the placentae and the ovules are excised aseptically, usually on the day of anthesis, and the pollen obtained aseptically is deposited on them. The method of application of pollen, the quantity and the kind of pollen used are dictated by the objectives of the experiment.

Once the incidence of fertilization in vitro is proven, the fertilized ovules, i.e. the incipient seeds, are usually transferred to a different nutrient medium evolved through experimentation to rear the young seeds to mature viable condition.

3. Results and Applications

Prompted by the success of intraovarian pollination (KANTA, 1960), the work was extended to investigate whether or not a pollination of excised ovules in vitro would also prove successful. Thus was the technique of in vitro pollination and fertilization devised by KANTA *et al.* (1962) of the University of Delhi; and while reporting it they suggested its possible applications in plant breeding and genetics. It is significant that for devising the technique *Papaver somniferum* was chosen, because in this species the pistil lacks a style and as such the length which the pollen tubes have to grow, from the stigma to the ovules, is short. But even this length was curtailed by excising the ovules under aseptic conditions and culturing them together with pollen grains in vitro. Consequent to such a test-tube pollination, pollen germination, growth of pollen tubes, entry of pollen tubes into ovules through the micropyle, fertilization, development of embryo and endosperm viable seed set and seed germination—all occurred in vitro (Fig. 1 A, B, D, F, H) as normally as in vivo. This success prompted the Delhi School to test the efficacy of this new technique with other plants, namely *Argemone mexicana* (Fig. 1 E), *Eschscholzia californica* (Fig. 1 C), *Dicranostigma franchetianum*, and *Nicotiana tabacum*. The technique proved a success with these plants as well (KANTA and MAHESHWARI, 1963; MAHESHWARI and KANTA, 1964; RANGASWAMY and SHIVANNA, 1969).


pollen
ovules
A
pollen grains
ovule
pollen tubes
B
pollen tube
pollen grain
ovule
C
D
E
embryo
endosperm
G
F
H

Logically, the next step was to investigate whether the in vitro technique could be modified to make it more similar to the situation in vivo. Therefore whole pistils, together with a short length of pedicel and calyx, were excised, suitably surface-sterilized, and implanted on agar nutrient media. The compatible pollen was also suitably rendered aseptic and then deposited on the stigma of the cultured pistils. Such stigmatic pollinations in vitro (Fig. 2 A–G)—cross-pollination in *N. rustica* (RAO, 1965; RAO and RANGASWAMY, 1972) and *Petunia violacea* (SHIVANNA, 1965), and self-pollination in *Antirrhinum majus* (USHA, 1965)—led to successful fertilization and seed set. Later viable seedlings were also raised. DULIEU (1963, 1966) also reported successful pollination and fertilization in pistil cultures of *N. tabacum*. These reports instigated further investigations in adapting and improving the basic technique formulated by KANTA *et al.* in 1962, and in applying it to problems of interspecific and intergeneric hybridizations, and self-incompatibility.

Working at the University of Delhi, ZENKTELER (1965) applied the test-tube fertilization technique to *Dianthus caryophyllus*. Later ZENKTELER and his co-workers extended this work and applied the technique to raise interspecific and intergeneric hybrids otherwise unknown in nature (ZENKTELER, 1967, 1970; GUZOWSKA, 1971; ZENKTELER *et al.*, 1975). They used *Melandrium album* and sometimes *M. rubrum* also as ovule parents. Ovules intact with the placentae were aseptically excised four days before, as well as on the day of anthesis, and cultured on WHITE'S (RANGASWAMY, 1961) or NITSCH'S nutrient agar medium (NITSCH, 1951). 15 species—*Cerastium arvense*, *Dianthus carthusianorum*, *D. serotinus*, *Lychnis coronaria*, *Melandrium album*, *M. rubrum*, *Minuratia laricifolia*, *Silene alpina*, *S. friwaldskyana*, *S. schafta*, *S. tatrica*, *Vaccaria pyramidata* (all of Caryophyllaceae), *Hesperis matronalis* (Cruciferae), *Datura stramonium* (Solanaceae), and *Campanula persicifolia* (Campanulaceae) were used as pollen parents. Pollen grains were collected under aseptic conditions from anthers excised a day before, or on, the day of anthesis and were spread on the cultured ovules. Cultures were grown for 4–14 days.

The pollen grains germinated within 2–12 h after culture in varying intensities. In all the combinations of the caryophyllaceous taxa, and in some interfamily combinations, pollen germination was much better on ovule surface in vitro than on the stigma in vivo. The pollen tubes did not follow any particular direction of growth on the ovule mass, and several of them entered the ovules through micro-

Fig. 1 A–H. In vitro pollination, fertilization, seed and seedlings formation in Papaveraceae. (A, B, D, F–H) *Papaver somniferum*, (C) *Eschscholzia californica*, (E) *Argemone mexicana*. (A) Portion of placenta with ovules (day of anthesis) cultured on Nitsch's agar medium and pollinated with mature pollen. × 3. (B) Wholemount from pollinated culture showing pollen germination and pollen tube growth on and around the ovule. × 51. (C) Wholemount from 1-day-old pollinated culture showing pollen tube entry into ovule; part of pollen tube inside micropyle is highlighted by 2 boundary lines in black. × 91.5 (D) 7-day-old culture; several young seeds (white bodies) are evident on the placenta. × 1.3. (E, F) Proembryo and globular embryo excised from seeds development in vitro. (E) × 635.25, (F) × 474.6. (G) Longisection of seed formed in 15 days from pollination and fertilization in test-tube; development of both embryo and endosperm matches well with that in field-borne seeds. × 66. (H) Fresh culture of seedlings raised from seeds removed from 22-day-old pollinated cultures. × 2.65. (A, C, E, G, H) after KANTA and MAHESHWARI, 1963; (B, D, F) after KANTA *et al.*, 1962

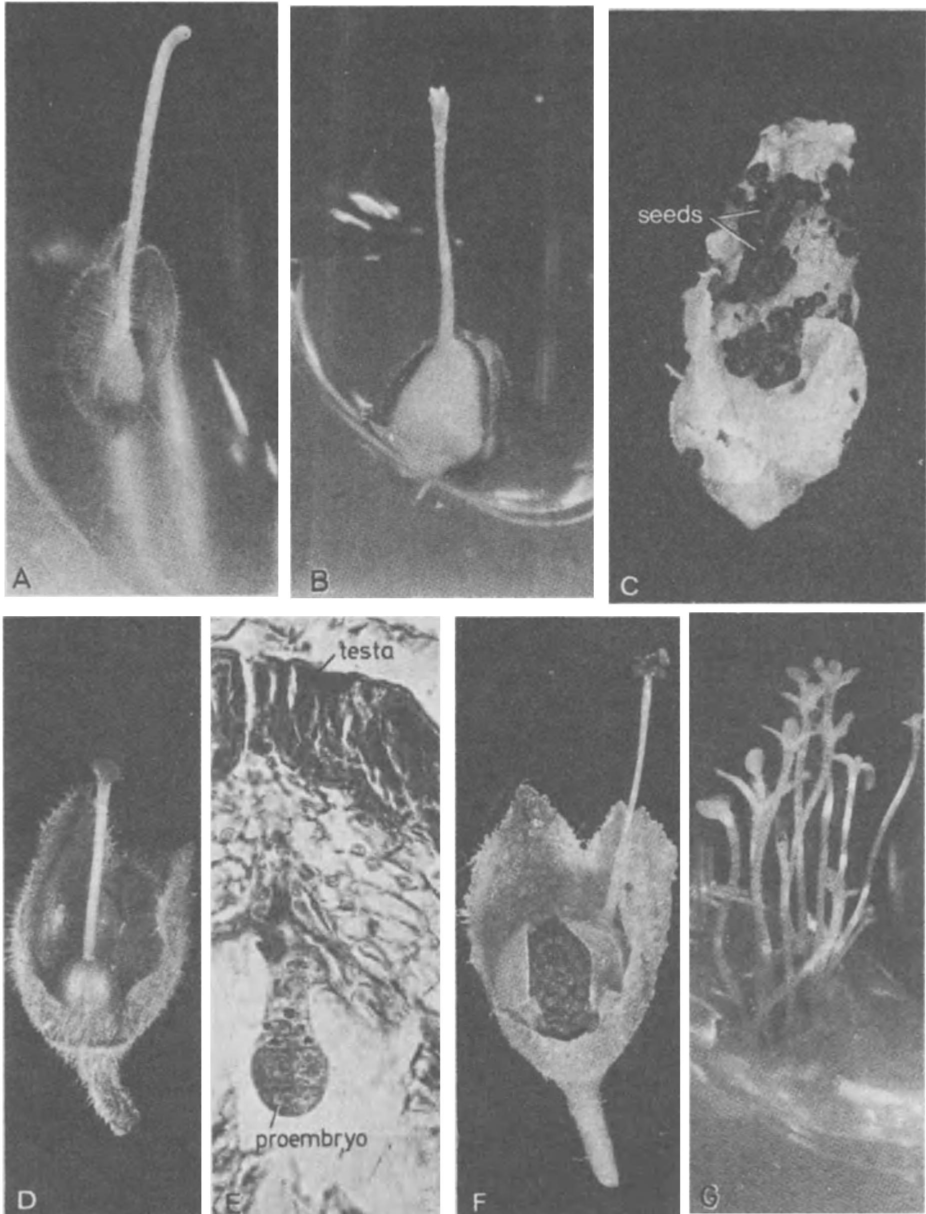


Fig. 2A–G. In vitro pollination, fertilization, seed set and seedling formation in pistil cultures. (A–C) *Antirrhinum majus*, (D–G) *Nicotiana rustica*. (A) Pistil culture ready for self-pollination. × 2.46. (B) 20-day-old self-pollinated culture showing fruit growth. × 1.65. (C) 31-day-old culture in which fruit wall is partially removed to show seed set in vitro. × 2.13. (D) Pistil made ready for culture and pollination. × 1.68. (E) Longisection through micropylar region of a seed from 10-day-old cross-pollinated pistil culture; note proembryo formation. × 437. (F) 21-day-old test-tube fruit showing dehiscence. × 2.5. (G) Pistil culture as seen 6 weeks after crosspollination. Note in situ seed germination; remnant of pericarp is visible at the hase of the bunch of seedlings. × 2.1. (A–C) after USHA, 1965; (D–G) after RAO and RANGASWAMY, 1972

pyle and effected fertilization. Ovules in which in vitro fertilization had occurred enlarged rapidly in two days after culture and underwent normal development into seeds containing viable embryos.

The most satisfactory development of hybrid embryos occurred in the crosses *Melandrium album* × *M. rubrum*, and *M. album* × *Silene schafta*. The resulting F_1 hybrids could be eventually raised to flowering in the field (ZENKTELER, 1967). In combinations of *M. album* with six other caryophyllaceous taxa tested—*Lychnis*, *Minuratia*, 3 species of *Silene*, and *Vaccaria*—both proembryo and endosperm were formed in 4 days of culture (GUZOWSKA, 1971; ZENKTELER *et al.*, 1975). Other intergeneric combinations (♀ *Melandrium*) resulted in embryologic aberrations (ZENKTELER, 1970; ZENKTELER *et al.*, 1975) such as bursting of pollen tubes, destruction of egg apparatus following pollen tube entry (♂ *Campanula*), degeneration of nucleus of egg cell, single fertilization leading to formation of either the proembryo (♂ *Campanula*, *Dianthus*) or the endosperm (♂ *Datura*, *Hesperis*), and degeneration of proembryo (♂ *Datura*). Further work must be directed towards improving the nutrient medium to obtain normal and complete development of the hybrid embryos.

Another field in which the technique of in vitro pollination and fertilization has found application is the overcoming of sexual self-incompatibility. For details on self-incompatibility see Chapter III.6 this Volume; however, a few remarks serve as necessary background. Self-incompatibility is a physiologic barrier which prevents fusion of sexually different gametes, which are otherwise fertile, produced by the same individual of a heterosporous species. In homomorphic flowering plants the barrier to selfing is usually expressed as inhibition of pollen germination on stigma (as in sporophytic system), or as inhibition of pollen tube growth in the style (as in gametophytic system). The technique of in vitro pollination and fertilization helps eliminate these incompatibility zones in bringing together the pollen and the ovule under aseptic conditions on artificial nutrient media. It should, however, be noted that both self-pollination in vitro on the stigma and pollination of excised ovules cultured in vitro have not helped overcoming self-incompatibility in some species tested (SHIVANNA, 1965). So the basic technique of test-tube pollination has been improved; the improved technique called "placental pollination" (as opposed to stigmatic pollination and isolated ovule pollination) proved a remarkable success in overcoming self-incompatibility in *Petunia axillaris* (RANGASWAMY and SHIVANNA, 1967, 1971a). In placental pollination, instead of pollinating isolated ovules or groups of ovules in vitro, the entire ovule mass of an ovary intact on the placentae is cultured and the pollen grains are liberally deposited all over the ovule mass (Fig. 3A). This technique helped retain in vitro the original (in vivo) arrangement of ovules on the placentae and thus prevented the surgical damage which seems to adversely affect the entry of pollen tubes into ovules. Subsequent to placental pollination the pollen grains germinated in 3 h, the pollen tubes grew luxuriantly among the bare ovules and on the placentae, and 1 day after culture the pollen tubes had already entered the ovules (Fig. 3B, C). Two days after culture many ovules had been fertilized (Fig. 3D, E) and the fertilized ovules, that is young seeds, showed embryo development (Fig. 3F) and were inescapably evident in the cultured ovule mass (Fig. 3G). Seven days after culture the young seeds turned mealy white and resembled seeds of the same age

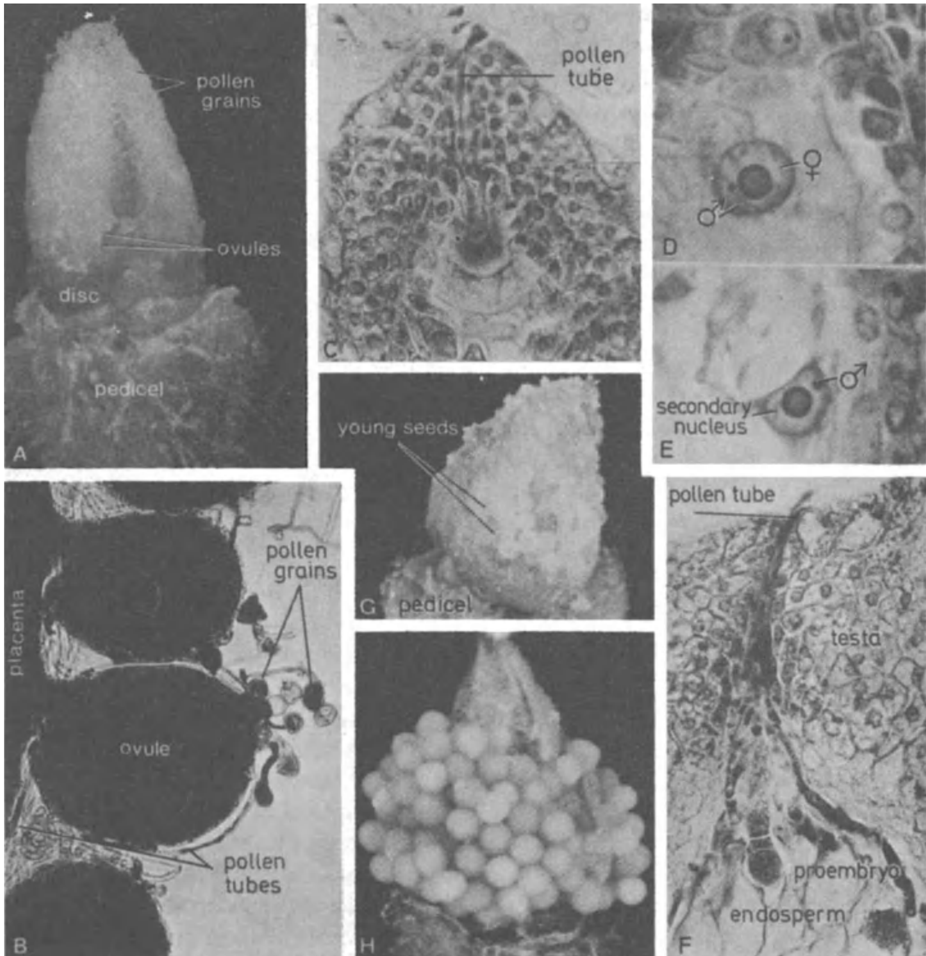


Fig. 3A–H. Placental pollination in vitro to overcome self-incompatibility in *Petunia axillaris*. (A) Both placentae of an ovary with its entire mass of ovules covered with pollen, and ready for culturing. × 10.37. (B) Free-hand transection through the self-pollinated placentae 24 h after culture; marginal portion of one of the placentae, 3 ovules, some pollen grains, several pollen tubes all over and forming a mesh especially along the placenta are seen. × 105. (C) Longisection through micropylar region of ovule 24 h after self-pollination in vitro; note entry of pollen tube into embryo sac. × 327.47. (D, E) Longisections of ovules 2 days after selfing in vitro; only micropylar half of the embryo sac is shown. (D) pictures syngamy and (E) triple fusion. (D) × 955, (E) × 951.2. (F) Longisection through micropylar region of seed resulted from placental self-pollination in vitro; observe the extramicropylar part of pollen tube, remnants of pollen tube in micropyle, testa, filamentous proembryo, and cellular endosperm. × 250. (G) 5-day-old culture showing enlargement of several self-fertilized ovules; unfertilized ovules are poorly visible. Placentae and pedicel also show general enlargement. Compare with (A). × 7.45. (H) Culture as seen 24 days after test-tube placental pollination. Numerous seeds have been formed. Of the unfertilized ovules barely anything is discernable on the placentae; instead that region of the placentae is now seen as a barren cone. × 7.6. (A, B, F–H) after RANGASWAMY and SHIVANNA, 1967; (C–E) after RANGASWAMY and SHIVANNA, 1971a

formed consequent to cross-pollination both in vivo and in vitro. Mature seeds containing a dicotyledonous adult embryo and normal endosperm were formed 24 days after placental self-pollination (Fig. 3 H). From the seeds formed in vitro due to placental self-pollination, F_1 plants were raised in the field. Like the parent, all the progeny was diploid, fertile, and self-incompatible. Remarkably, self-incompatibility was overcome in the F_1 as well through the same technique of placental pollination. *Petunia hybrida* is yet another species in which self-incompatibility has been overcome by in vitro pollination and fertilization (NIIMI, 1970).

The pioneering success of overcoming self-incompatibility in placental pollination (RANGASWAMY and SHIVANNA, 1967) has opened rewarding vistas of research in plant breeding. For example, one could introduce in vitro a mechanical barrier, such as a piece of cellophane or mica sheet, between the placentae of the same ovary and treat the ovules on the different placentae differently. Working with the self-incompatible species *P. axillaris*, RANGASWAMY and SHIVANNA (1971a) introduced cellophane between the two placentae (Fig. 4 A) and then gave the following three sets of differential treatments to the placentae: (1) one of the placentae was self-pollinated and the other was cross-pollinated (selfed vs crossed), (2) one placenta was self-pollinated and the other was left without pollination as control (i.e. selfed vs control), (3) one placenta was cross-pollinated and the other was left as control (i.e. crossed vs control). All the operations were done under aseptic conditions and the cultures were raised as described earlier. The control placentae invariably shrivelled (Fig. 4B), whereas the pollinated placentae (whether selfed or crossed) showed all the embryologic activities that occur consequent to successful pollination. In 21 days of culture mature seeds were obtained on the pollinated placentae (Fig. 4 B–D). The most striking result of differential pollinations in vitro is that there was no difference in the degree of seed set between the selfed placenta and the crossed placenta (Fig. 4 C); the positive correlation between them was of a high degree and the *t* value was significant at 5% level (RANGASWAMY and SHIVANNA, 1971 a).

That placental pollination could be successfully applied to overcome self-incompatibility in *P. axillaris* (RANGASWAMY and SHIVANNA, 1967, 1971 a) raised an important query: if stigmatic self-pollination and placental self-pollination could both be performed in the same pistil, would stigmatic self-pollination nullify placentae, and the pistil cultured (Fig. 4 E). The thus exposed placenta and the placental self-pollination have a salutary effect on stigmatic self-pollination? To find answers to these queries, RANGASWAMY and SHIVANNA (1971 b) devised a method of 2-site pollinations in pistil cultures of *P. axillaris*. After surface-sterilizing the pistil, the ovary wall was carefully peeled to expose only one of the two placentae, and the pistil cultured (Fig. 4e). The thus exposed placenta and the stigma were both used for in vitro pollinations as desired; as many as nine pairs of treatments separated in time were studied. In the treatment pair which comprised successive self-pollination at both sites (stigma and exposed placenta), the pollen tubes were inhibited in the style, and seeds invariably developed on the exposed placenta (Fig. 4 F, G) and seldom on the covered placenta. The result was similar when self-pollination at one of the two sites was delayed by 2 days in reference to the pollination at the other site (Fig. 4 H). Results of all other treatments also demonstrated the stigmatic self-pollination (in *P. axillaris*) to be incompatible, no

matter what treatment was given the exposed placenta (Fig. 41), and the placental self-pollination to be invariably successful.

WAGNER and HESS (1973) utilized the technique of 2-site pollinations, again for *Petunia*, to study the relative fertilization competence of pollen grains (presumably compatible) deposited on the stigma and those deposited on the ovule surface. They used two pure lines of *P. hybrida*, the cyanidin type and 34d10. They observed that the pollen put on the stigma had a much better chance of effecting fertilization, despite the longer way their pollen tubes had to traverse, than the pollen deposited on the ovules.

A third application of in vitro pollination is in inducing the formation of haploid plants. Haploid sporophytes are invaluable in plant breeding because by a mere doubling of their chromosome number homozygous diploids can be derived. Although various techniques such as delayed pollination, distant hybridization, "pollination" with abortive pollen or with irradiated pollen, physical and chemical treatments to the ovary (MAHESHWARI and RANGASWAMY, 1965), and recently anther culture (Chap. II.1 of this Vol.), have been adopted to obtain haploids, induction of haploid sporophytes is still a rare and uncontrolled phenomenon. Recently HESS and WAGNER (1974) reported that their attempts to establish haploids of *Mimulus luteus* (Scrophulariaceae) through anther culture were negative, but when its ovule mass was pollinated in vitro with pollen of *Torenia fournieri* (also of Scrophulariaceae) there resulted 1% haploid plantlets in the cultures. These plantlets were successfully transplanted to soil. That these plantlets were not *Mimulus* × *Torenia* hybrids but were haploids of the ovule parent *Mimulus* is claimed on the basis of chromosome counts, anthocyanin content of petals, and the size of various parts (including the isolated protoplasts), all of which were half the size of those from normal diploid *Mimulus*.

Besides its practical applications chiefly in plant breeding and genetics the technique of in vitro pollination and fertilization has been employed to study some problems of pollen physiology and fertilization (KAMEYA *et al.*, 1966; BALATKOVÁ and TUPÝ, 1968). For example, it is rather difficult to achieve in vitro germination of pollen of Cruciferae, and consequently fertilization in vitro is also refractory. Because calcium has been reported to favour the chemotropic growth of pollen tubes towards ovules (BREWBAKER and KWACK, 1964), KAMEYA *et al.* (1966) excised the ovules of *Brassica oleracea* a day before anthesis, dipped them in a 1% solution of calcium chloride, then sowed them on a gelatin film, and immediately applied to them the pollen collected from fresh flowers. Next day such ovules as had received a pollen tube were transplanted under aseptic conditions to NITSCH's medium (NITSCH, 1951). Only a couple of seeds were formed in 3 months. Although this seed number is negligible, this work suggests that a suitable pretreatment of ovules may prove helpful for achieving in vitro fertilization.

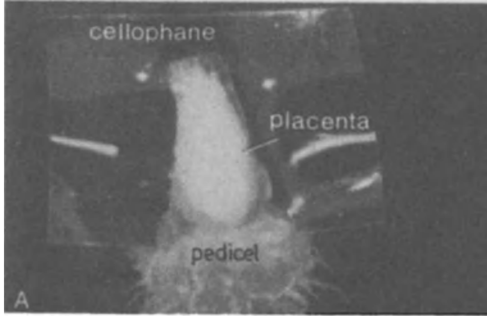
BALATKOVA and TUPÝ (1968) utilized the technique of test-tube fertilization to investigate whether artificial cultures of pollen tubes can effect fertilization and whether the pollen tubes retain the fertilizing ability even after gamete formation. They chose *Nicotiana tabacum* for this investigation. Under aseptic conditions the ovary (1 day after anthesis) was split longitudinally to isolate the two placentae which were later pollinated in vitro. For pollinating the placentae, instead of

pollen grains, pollen tube cultures were used in three ways: (1) part of a pollen tube culture reared in a pollen germination liquid medium was lodged by a glass rod on to the cultured placentae; (2) the surface of the nutrient agar medium meant for placental culture was wetted with pollen germination liquid medium, pollen was dusted on it, and after the desired time interval the excised placenta was implanted on the resulting pollen tube culture; and (3) pollen was dusted on small squares of cellophane which had been placed on a semi-liquid pollen germination medium, the cellophane square bearing the mat of pollen tubes was first implanted on the medium meant for placental culture and then the placenta was placed on the cellophane mat of pollen tubes. In *N. tabacum* male gametes were formed in the pollen tube from 10 to 18 h after pollen culture; therefore 8-h-old and 20-h-old pollen tube cultures were used for pollinating the placentae. All three methods of placental pollination by pollen tube cultures successfully led to fertilization and seed set; the pollen tubes could effect fertilization even after gamete formation in them (20-h-old cultures). In their ability to cause seed set the decreasing order of the three methods was (2), (1), and (3). These methods offer the advantage of giving only the male gametes (in pollen tubes) any desired treatment prior to the use of pollen tubes for in vitro pollination.

The extent to which a technique can be put into practical use is usually governed by the number and nature of the parameters that affect the operation of the technique and the degree of easiness with which the parameters can be controlled. The index for the success of test-tube fertilization technique is the degree of viable seed set. And this is dependent on (1) age of the explants, particularly the ovules, (2) adequate pollen germination, (3) proper growth of pollen tubes and microgametogenesis, (4) pollen tube entry into ovules, (5) high incidence of fertilization, and (6) development of viable seeds.

Little is known about the effects of physical factors such as light, temperature, and humidity on seed set consequent to test-tube fertilization. As regards light, it is reported that its presence or absence during the culture period does not seem to affect seed set in vitro. Working with *N. tabacum*, BALATKOVÁ and TUPÝ (1972) found that the best seed set was achieved with the placentae excised 1 and 3 days after anthesis; explants 1 day before anthesis and 5 days after anthesis were less efficient.

In achieving pollen germination (on placenta or ovule surface in vitro) a major precaution, seldom recorded in the literature but usually practised by the investigators, is to avoid any film of water on the surface of ovules or placentae. The method of application of pollen on to the surface of ovules is also a factor which affects seed set in vitro. In *N. tabacum*, for example, application of pollen on to a specific locus of the placenta improved seed set more substantially than deposition of pollen over the surface of the entire ovule mass or sowing pollen on the nutrient medium in close proximity to the placenta. This in turn is presumably related to the factor that a certain period of pollen tube growth must elapse for microgametogenesis to occur. Depositing the pollen at a specific site on the ovule mass thus permits a distance and a period of growth for the pollen tubes to cover before the generative cell can produce the male gametes, and prevents the formation of a dense mat of pollen tubes which would result from the dusting of pollen all over the ovule mass (BALATKOVÁ and TUPÝ, 1972). That pollination of excised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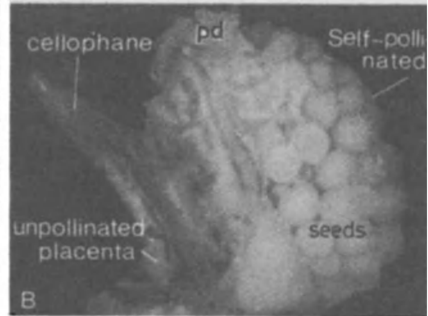
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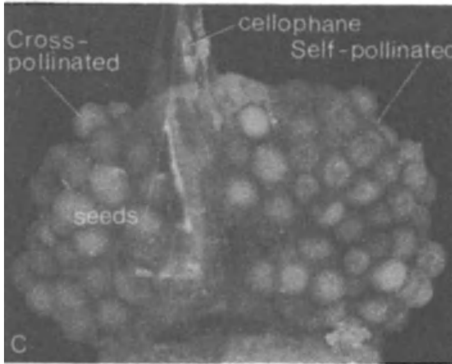
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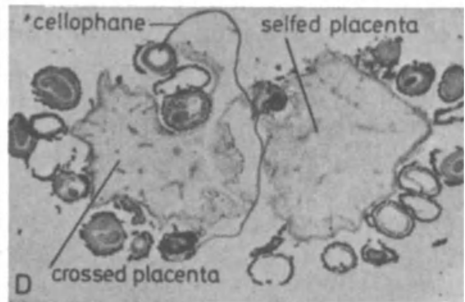
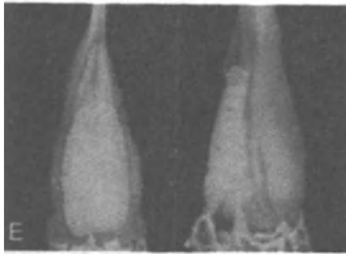

cellophane
selfed placenta
crossed placenta
D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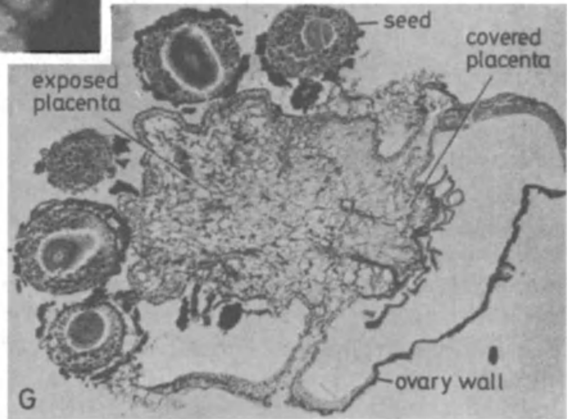
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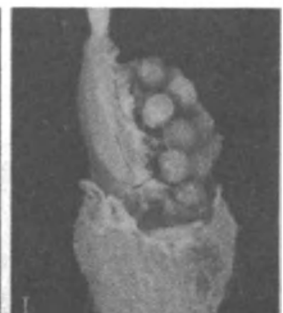
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ovules has rarely led to successful fertilization, unlike the pollination of whole ovule mass intact on the placenta(e), suggests that mechanical injury caused to ovules during their excision from the placenta seem to affect pollen tube entry into ovules adversely.

Besides the factors related to fertilization, the success of test-tube fertilization is affected also by factors which govern the development of fertilized ovules into seeds, and of zygotes into mature viable embryos. In this connection the composition of the nutrient medium seems to play a cardinal role. For example, both MAHESHWARI and KANTA (1964) and BALATKOVÁ and TÜPÝ (1972) demonstrated that supplementing the culture medium with casein hydrolysate (500 mg/l) favoured in vitro seed development in *Papaver somniferum* and *N. tabacum*, respectively. DULIEU (1966), however, reported its less salutary effect on embryo growth in cultures of fertilized ovules of *N. tabacum*. RANGASWAMY and SHIVANNA (1971a) also found that casein hydrolysate (same concentration) did not promote or suppress seed set in placental pollinations of *Petunia axillaris*. For it to be useful in plant breeding test-tube fertilization must necessarily lead to proper embryo growth in the resultant seeds. Because embryo growth and development are governed by critical shifts in hormonal balance at various stages (RANGASWAMY, 1963; MAHESHWARI and RANGASWAMY, 1965; RAGHAVAN, 1966; REINERT, 1973), it may be worthwhile to isolate the ovules fertilized in vitro and culture them on a further modified medium to obtain viable seeds; this should prove especially rewarding in instances of embryo degeneration in test-tube hybridization, for example *Melandrium* × *Datura* (ZENKTELER, 1970).

Fig. 4A–I. Test-tube pollination and fertilization in *Petunia axillaris*. (A–D) Differential treatments of placentae in vitro. (A) Explant after insertion of cellophane between the placentae, as seen in face view of one of the placentae. Contours of several ovules are clear. × 6.44. (B) Placental culture as seen 14 days after differential treatments—unpollinated (control) placenta at left, self-pollinated placenta at right. Unpollinated placenta is seen as a shrivelled mass adpressed to the crumpled cellophane. On the self-pollinated placenta several seeds have been formed; note also remnant of the pollen deposit (*pd*). × 12.18. (C) 21 days after differential treatments—cross-pollinated placenta at left and self-pollinated placenta at right. Both placentae bear numerous seeds consequent to pollination and fertilization in vitro. Cellophane has folded over the cross-pollinated placenta. Compare with (B). × 15.16. (D) Transection through approximately the middle region of the culture shown in (C). As in (C), to the left is the crossed placenta and to the right the selfed placenta; cellophane between them is seen as a black thread coiled toward crossed placenta. On both placentae seeds cut in favourable planes show the embryo. × 12.98. (E–I) Two-site pollinations in vitro. (E) Pistils made ready for 2-site pollinations by removing ovary wall on one of the 2 placentae. *Left*—exposed placenta in face view, *right*—exposed placenta in profile (note also the covered placenta with the ovary wall intact). × 4.49. (F) 21-day-old culture in which exposed placenta and stigma had been both self-pollinated successively on day of culture; seed set occurred on exposed placenta only. × 4.48. (G) Transection approximately through middle region of the placentae shown in (F). Note that covered placenta (*right*) shows all ovules degenerated, and exposed placenta shows formation of many seeds containing embryo and endosperm. × 24.53. (H) 24-day-old culture in which the exposed placenta (*left*) was self-pollinated on day of culture, and the stigma 2 days later; only the exposed placenta showed seed set × 4.5. (I) 21-day-old culture in which the stigma was self-pollinated and the exposed placenta (*right*) cross-pollinated, successively on day of culture; seed set is obvious on exposed placenta only. × 4.1. (A–D) after RANGASWAMY and SHIVANNA, 1971a; (E–I) after RANGASWAMY and SHIVANNA, 1971b

4. Summary and Conclusions

Although the technique of test-tube fertilization is rather recent (KANTA *et al.*, 1962), its usefulness in applied and fundamental botany has already been notable. It has great potential and its prospects are wide open. The placental pollination method, for example, should prove promising in those systems in which selfing or hybridization has not been successful, due to either the inhibition of pollen germination on the stigma (particularly even after treating the stigma) or the inability of pollen tubes to reach the ovary. Additionally, in studies of induced parthenogenesis and in mutation research, placental pollination provides a better control and a means of treating only the female partner (ovules), because the technique facilitates a direct deposition of the desired kind of pollen or chemical mutagens in definitive loci on the ovule mass. Likewise, the use of pollen tube cultures for in vitro pollination offers a convenient method of treating only the male partner (gametes in pollen tubes) with physical and chemical mutagens.

In eusyncarpous pistils the stigmatoid tissue through which the pollen tubes traverse is common to all the carpels. Consequently, pollen tubes issued by pollen grains deposited at any point on the stigma can fertilize ovules borne on any of the placentae. Thus, in experimental studies on the pistil the entire ovary, and theoretically att its ovules, become subjected to a single treatment. Hence, any manipulation which would permit differential treatments of the placentae in the same pistil, and thus very effectively reduce the sample variation to the minimum, is of special value in studies of pollination and sexual incompatibilities. In this direction the techniques of placental pollination and of introducing a cellophane piece along the septa (RANGASWAMY and SHIVANNA, 1967, 1971a; SHIVANNA, 1971) are important. Likewise, test-tube fertilization technique is presumably a more rewarding substitute to delicate operations such as style grafting, tandem grafting, and stump pollination, which are not always easy to accomplish, nor always successful in field conditions.

In view of the recent success of HESS and WAGNER (1974) in obtaining haploid plants through in vitro pollination technique but not through anther culture, the application of the test-tube pollination technique to instances in which anther culture has been a failure may prove rewarding. By in vitro pollination the pollen grains with their store of growth-promoting factors are brought into much closer contact with the embryo sacs than in other pollination methods, and this may favour instigating the egg cell to undergo parthenogenesis.

Indeed, in the technique of test-tube fertilization the plant breeder has a powerful tool to help him realize his aspirations of rearing distant hybrids and homozygous plants much more easily than has been possible until now. This technique, coupled with the recent "recognition pollen" method which has proved a success in obtaining interspecific hybrids (KNOX *et al.*, 1972) and in inducing self-compatibility (HOWLETT *et al.*, 1975), should prove doubly valuable for studies on systems in which manual pollination in vivo is not easy to achieve. Hybridization of cultivated crop plants with their putative parents and wild relatives is basic to studies on crop improvement; test-tube fertilization should prove useful in such studies.

The value of in vitro pollination and fertilization as a technique in fundamental fields such as ovule physiology before, during, and after fertilization cannot be overemphasized. To employ this technique to raise hybrids between the two groups of seed plants, the gymnosperms and the angiosperms, would be an interesting vocation, and may throw light on such problems as the origin of flowering plants!

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References see page 442.

6. Incompatibility and in vitro Cultures[1]

D. DE NETTANCOURT and M. DEVREUX

1. Introduction

Sexual incompatibility is represented by a group of mechanisms which control, in numerous species of higher plants, the upper and lower limits of genetic exchanges. On the one hand, the phenomenon establishes panmixis by preventing self-fertilization to occur in a majority of allogamous plants *(self-incompatibility)* while, on the other hand, it attenuates or forbids the flow of genes between populations *(interspecific incompatibility)*. In other words, sexual incompatibility favors gene circulation within the population but stimulates speciation and the reinforcement of reproductive isolation between distinct populations.

In the first part of this review, it is proposed to define briefly the main features of these two different types of sexual incompatibility and, àt the same time, to outline, whenever appropriate, the contributions which in vitro researches have made to our knowledge on incompatibility phenomena. In the last sections, special attempts are made to review the various possibilities, potentially enormous but still very restricted at the moment, which arise from the conjunction of researches on incompatibility and of the use of in vitro culture techniques.

2. Self-incompatibility

As several reviews are available on the subject (see LEWIS, 1949, 1960; BATEMAN, 1952; PANDEY, 1959; LUNDQVIST, 1965; LINSKENS, 1965; ARASU, 1968; LINSKENS and KROH, 1970; DE NETTANCOURT, 1972; DICKINSON and LEWIS, 1973a, b; HESLOP-HARRISON *et al.*, 1974), only the general aspects of the system and the specific characteristics which are of direct importance for tissue or cell culture researches will be mentioned in this report.

2.1 The Definition of Self-incompatibility

The literature is unclear as to whether or not the use of the term "self-incompatibility" must be restricted to pre-fertilization processes or extended to describe all events which prevent fertile hermaphrodites from setting seeds upon selfing. Simi-

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larly, a certain confusion sometimes arises between sterility, which, before fertilization, is independent of the mating partners involved, and incompatibility, where both the pollen and the pistil participate in a complex recognition process.

In view of very basic differences in function, mechanism, and occurrence between post- and pre-zygotic barriers and between sterility and incompatibility *per se*, it seems, however, most advisable to adopt the attitude of LUNDQVIST (1964) and to define self-incompatibility in higher plants as "the inability of a fertile hermaphrodite seed-plant to produce zygotes after self-pollination".

2.2 Classification of Self-incompatibility Systems

Although, with few possible exceptions, all self-incompatibility systems are based upon the fact that the incompatibility reaction occurs each time pollen and pistil carry identical incompatibility substances, there exists in flowering plants a diversity of incompatibility systems which have been classified on the basis of the five following parameters:

2.2.1 The Time of Gene Action

In all incompatibility systems, the time of gene action in the pistil, that is to say the stage at which the host tissue of the stigma or the style determines its capacity to reject incompatible pollen, coincides with the opening of the flower. In a regular diploid species, two alleles, for each incompatibility locus involved, participate in this determination via an independent action of each allele or through relationships of dominance or interactions.

In contrast with the situation in the pistil, the stage at which the male gametophyte receives the information necessary for the determination of its incompatibility phenotype can greatly vary from one family to the next and is one of the essential features upon which the classification of self-incompatibility systems is founded. All types of incompatibility can, on this basis, be subdivided into two distinct groups:

Sporophytic Incompatibility. Here the incompatibility phenotype in the pollen is determined by the genotype of the pollen-producing plant. As for the pistil, two alleles in a diploid individual participate, for each incompatibility locus involved, in this determination which can be characterized by independent action of each allele or through relationships of dominance or interactions.

Gametophytic Incompatibility. The genotype of the individual microspore determines the phenotype of the pollen. In such a system, interactions or dominance relations between alleles of a same locus are impossible in the pollen and, since self-incompatibility is the result of identical phenotypes in pollen and style, they are also lacking in the pistil where each allele has independent action. Interactions between alleles of different loci can and do occur, however, in the pollen and the pistil of species, such as those of the Graminaceae family (LUNDQVIST, 1962), which are characterized by a multifactorial system of self-incompatibility.

2.2.2 The Association with Floral Polymorphism

In certain species characterized by sporophytic incompatibility, there is an association between the morphology of the flower and its incompatibility phenotype. The associated characteristics do not only concern cell size, stylar length, and anther levels but also, at least in certain cases, osmotic pressure and the sculpturing of the pollen exine.

2.2.3 The Site of Expression

Depending upon the species and the system involved, the inhibition of incompatible tubes may take place on the stigma, in the style, or in the ovary. As a rule, stigmatic inhibition occurs in species with tri-nucleate pollen (BREWBAKER, 1957) and ovarian inhibition characterizes plants with hollow style. The two phases, recognition and rejection, which, as shall be seen later, characterize the incompatibility reaction, do not necessarily involve similar sites.

2.2.4 Polyallelic Series

In gametophytic systems and in some homomorphic sporophytic species, very large numbers of incompatibility alleles are segregating at the incompatibility locus (S-locus). Hundreds of different S-alleles have been, for instance, recorded or estimated in small populations of *Oenothera* (EMERSON, 1939) and *Trifolium* (ATWOOD, 1944; WILLIAMS and WILLIAMS, 1947). This extreme diversity in the allelic state of a single locus is, of course, of considerable interest for the evaluation in vivo or in vitro of gene structure and the analysis of gene products.

2.2.5 Number of Loci

Although a single locus controls self-incompatibility in a large number of allogamous species, cases are known where the reaction is governed by two or more loci (see, for instance, the situation in *Lythrum* [VON UBISCH, 1921]; in *Capsella* [RILEY, 1936]; the grasses and sugar-beets [LUNDQVIST, 1956; LUNDQVIST *et al.*, 1973]. CROWE (1971) has even discovered, in *Borago officinalis*, a very unusual post-zygotic mechanism of self-incompatibility which is governed by polygenes.

3. The Distribution of Self-incompatibility Systems

Several reviews and surveys have been made on the distribution of self-incompatibility in flowering plants (EAST, 1940; BREWBAKER, 1959; VUILLEUMIER, 1967) which show that the phenomenon occurs in at least 6000 species distributed in 250 genera for 70 families representing more than 19 orders of monocotyledoneae and dicotyledoneae. Leguminosae and Solanaceae are usually characterized by what is considered the ancestral basis of self-incompatibility, that is to say the gametophytic monofactorial system. Graminaceae generally display bi-factorial

gametophytic control while Crucifereae and Compositae exhibit the more recent sporophytic–homomorphic system. Linaceae, Lythraceae, and Oxalidaceae are heteromorphic.

4. Morphology of the Self-incompatibility Reaction

Depending upon the system involved and the type of incompatible mating performed, the self-incompatibility reaction may lead to three distinct events which are the inability to germinate, pollen tube growth inhibition, or fertilization failure.

4.1 Germination Failure

Germination failure only occurs when a stigmatic barrier is involved, but is not necessarily typical of all systems with stigmatic inhibition because in many instances the pollen germinates and penetrates the outer layers of the stigmatic papillae. A clear example of incompatibility leading to germination failure has been described by LEWIS (1943) and by DICKINSON and LEWIS (1974) in *Linum grandiflorum*, a species composed of two types of plants, pin (with long anthers and short styles) and thrum (with short anthers and long styles). Compatible crosses only occur between pin and thrum individuals; pin pollen, when applied on a pin stigma, completely fails to germinate, to swell, or to display any morphological change detectable by the electron microscope. LEWIS (1943) showed that this absolute passivity of pin pollen on a pin stigma resulted from the fact that the ratio of the osmotic pressures in pollen and stigma was too low to allow any transfer of water from the style to the pollen.

A similar situation is possibly representative of the homomorphic polyallelic mechanism of the Cruciferae as DICKINSON and LEWIS (1973a) found in *Raphanus* that a fraction of the pollen population fails to germinate and to undergo any visible cytoplasmic evolution or ultrastructural change after deposition on an incompatible stigma. However, one cannot discard the possibility that some kind of interaction is initiated between the incompatible pollen grain and the protein coat detected by MATTSSON *et al.* (1974) on the surface of the stigma papillae and suspected to serve, in the Cruciferae, as a primary recognition site for the inhibition of tube growth in the fraction of incompatible pollen grains which are able to germinate on an incompatible stigma.

4.2 Growth Inhibition

In this case, germination proceeds normally but the pollen tube is inhibited in its growth. The cessation of growth may, as in the Cruciferae, involve the inability of the incompatible tube to produce or activate the cutinase necessary for penetrating the stigmatic cuticle (KROH, 1964; LINSKENS and KROH, 1967; KROH and

MUNTING, 1967), or occur further down, always as a result of a recognition event at the time of germination, in the stigma or in the style.

In the sporophytic polyallelic system of the Cruciferae, where a proportion of the incompatible pollen not only manages to germinate, but also by-passes the cuticle barrier, such inhibition is accompanied by the deposition of callosic layers in the papillae a few hours after pollination (DICKINSON and LEWIS, 1973a). The generation of these callosic bodies involves the interaction of products of the stigmatic cytoplasm with substances produced by the incompatible tubes, but nothing is known with regard to the factors bringing about the inhibition of pollen tube growth. The cytoplasm of the pollen tube does not display any evident morphological change under the electron microspore.

In gametophytic monofactorial systems such as those of the Solanaceae, pollen tube growth generally proceeds unimpaired through the first third of the style. Then, the tip of the incompatible pollen tube bursts open after the outer wall has considerably expanded in the intercellular spaces of the conducting tissue and the inner-wall has disappeared. The tube cytoplasm, at this stage, is filled with bipartite particles measuring approximately 0.2 μm in diameter and giving a weak reaction to the test of Thiéry (DE NETTANCOURT *et al.*, 1973). The endoplasmic reticulum appears as a whorl of concentric layers which is perhaps indicative of a general cessation of protein synthesis in the incompatible tube (DE NETTANCOURT *et al.*, 1974).

In the bifactorial gametophytic system of grasses, the pollen usually germinates but, as observed by HAYMAN (1956) by means of the cotton-blue staining technique, the pollen cytoplasm fails to be released in the pollen tubes which are rapidly inhibited in their growth. Working with *Phalaris tuberosa*, KNOX and HESLOP-HARRISON (1971) followed, by means of immunofluorescence techniques, the fate of the intine-held antigens in compatible and incompatible pollinations and found that they diffused on the stigma, regardless of any subsequent germination, within the first minutes following pollination. The involvement of these antigens in the incompatibility reaction appears obvious in view of the results obtained by LEWIS *et al.* (1967), who showed, for the monofactorial gametophytic system of *Oenothera*, that the antigen associated to any S-allele could be detected by a precipitin reaction around the pollen grain carrying it when this pollen grain was placed on an agar medium containing an antiserum for a pollen extract of the same genotype.

4.3 Fertilization Failure

In addition to the classical case of *Theobroma cacao* (KNIGHT and ROGERS, 1955; COPE, 1958; BOUHARMONT, 1960), a number of examples are known of incompatibility reactions occurring in the ovary shortly before syngamy or after penetration of the pollen tube in the ovule. These often concern plant genera with hollow styles such as *Annona*, *Gasteria*, *Hemerocallis*, *Lilium*, *Narcissus*, *Ribes* (BATEMAN, 1954; ARASU, 1968) and sometimes occur after fertilization, during the first division of the endosperm in *Gasteria* (SEARS, 1937) or during embryonic growth in *Borago* (CROWE, 1971).

5. Biochemistry of Self-incompatibility

5.1 The Site and Timing of S-gene Activity in the Sporophytic System

The elegant experiments carried out by DICKINSON and LEWIS (1973a,b) and HESLOP-HARRISON *et al.* (1973) have shown that, in the Liliaceae, Cruciferae, Malvaceae, and the Compositae, the pollenkitts (colored coating of the pollen grains which contain carotenoids) and the tryphines (heterogeneous coatings consisting of proteins and lipids) originate from the tapetum during microsporogenesis and accumulate in the cavities of the sculptured exine. A fibro-granular component of the tryphine is essentially composed of protein and could be observed to be released through cisternae of the endoplasmic reticulum. In *Iberis*, HESLOP-HARRISON *et al.* (1974) clearly showed, through the use of the fluorescent protein "probe" I—anilinonaphtylsulphonic acid (I-ANS)—that the migration process was initiated some 60–70 h before anthesis, when the dissolution of the tapetum begins. These scientists were able to demonstrate, by means of transfer techniques which should greatly interest the botanists specializing in in vitro studies, that extracts of the tryphine accumulated around the pollen grain could induce the typical symptoms of the incompatibility reaction.

DICKINSON and LEWIS (1973a, b) showed, in this connection, that the tryphine extracted from *Raphanus* pollen penetrates the outer layers of a stigma and, whenever the pollen source is incompatible with this stigma, stimulates below the stigmatic papillae the production of the callosic body, which is typical of the incompatibility reaction. Working with *Iberis*, HESLOP-HARRISON *et al.* (1974) induced the rejection reaction in two different ways, with agar or agarose gels into which pollen-wall material had been allowed to diffuse and, also, with isolated fragments of the tapetum itself taken from anthers of incompatible plants. Using thin-layer chromatography and partial fractionation of the exine-held materials, it was possible to show that the exine proteins or glycoproteins, which induce the incompatibility reaction, belong to fractions with molecular weights between 10000 and 25000 daltons. It is probable that similar incompatibility proteins are formed in the pistil and that the recognition after self-pollination of identical gene products in the exine and in the pistil initiates the incompatibility reaction or, at least, its recognition phase.

5.2 The Activity of the Incompatibility Gene in the Gametophytic System

The indirect evidence that the self-incompatibility reaction involves gene activity for its initiation or its completion essentially stems from experimental observations which show:

- Specific differences in the patterns of stylar proteins after cross- and self-pollinations (LINSKENS, 1955).
- A rapid consumption of proline in self-pollinated styles of *Oenothera missouriensis* (LINDER and LINSKENS, 1972).
- The production of proteins which, as shown by serological tests (LEWIS, 1960; LINSKENS, 1960; MÄKINEN and LEWIS, 1962) or electrophoretical separations

(PANDEY, 1967; for a discussion, see BREDEMEIJER, 1974, and for the sporophytic system of *Brassica*, NASRALLAH and WALLACE, 1967; NASRALLAH *et al.*, 1969), are specific to each individual S-allele.

— The sensitivity of the incompatibility reaction to inactivation by heat treatments (HECHT, 1964), acute irradiation (LINSKENS *et al.*, 1960; HOPPER and PELOQUIN, 1968), or to mutagenic changes (LEWIS, 1951; PANDEY, 1956, 1967; VAN GASTEL and DE NETTANCOURT, 1975) which often correspond to deletions or injuries to the regulating elements of the S-gene.

More direct and more conclusive proof that S-gene activity controls the incompatibility reaction has been recently presented by VAN DER DONK (1974) who found that:

— RNA and protein syntheses occur earlier in self-pollinated styles than in cross-compatible ones.

— The RNA responsible for the main differences between self- and cross-pollinated styles is messenger RNA (the messenger activity of this RNA was tested through injections, together with ^{3}H-leucine, in egg cells of the tadpole *Xenopus levis*. After incubation, the proteins were extracted and the specific incorporation calculated). In other words, differential gene activity is quantitatively associated to compatible and incompatible tube growth.

— Protein patterns differed in self- and cross-pollinated styles and were not the same in all stages of pollen tube—style interactions (conclusions reached by VAN DER DONK through experiments where the messenger RNAs of cross- and self-pollinated styles were injected into *Xenopus* egg cells together with ^{3}H-leucine and the proteins separated by SDS-ureum-polyacrylamide gel electrophoresis). In other words, the rejection or acceptance of pollen tubes by styles is a complex process which, after the initial recognition reaction, appears to involve the activation and inactivation of several different genes.

5.3 Postulated Incompatibility Enzymes and Reaction Models

Although several reports suggest the involvement of enzymes in the self-incompatibility reaction (for a review see KROES, 1973, and BREDEMEIJER, 1974), little is really known about the nature of these enzymes among which the most likely ones appear to be:

— The cutinase, detected by LINSKENS and HEINEN (1962) in the Cruciferae (sporophytic system) and shown by these authors to enable the pollen to germinate through the stigmatic cuticle and to be lacking in incompatible pollen. A recent work by DICKINSON and LEWIS (1973a, b) does indicate, however, that the "cutinase mechanism" alone cannot explain self-incompatibility and that further research is still needed for understanding the incompatibility barrier of Cruciferae.

— Peroxidase isoenzymes, reported by PANDEY (1967) to be S-allele specific (for a recent evaluation and a discussion of the possible role of peroxidase isoenzymes in the self-incompatibility reaction, see also BREDEMEIJER, 1973, 1974).

— Cytochrome oxidase, amylase and acid phosphatase which SCHLÖSSER (1961) found to have a high activity in incompatible pollen tubes.

— Glycan hydrolases which LINSKENS *et al.* (1969) observed to increase after cross-pollination and to remain at low activity in self-pollinated styles.

Such information does not allow the elaboration of any conclusive model of the self-incompatibility reaction and does not even permit to find out if the rejection process results from an inactivation of pollen growth or from a mere absence of growth promotors (for a discussion, see KROES, 1973; DE NETTANCOURT *et al.*, 1974; LINSKENS, 1975).

It appears reasonable, at the present stage, to adhere to the model of LEWIS (1965) which is consistent with most of the facts known to date and specifies that the reaction of self-incompatibility results from the recognition of identical gene products in pollen and style which polymerize to form the incompatibility complex. This incompatibility complex may possibly act as a growth inhibitor to repress further evolution of the pollen tube or, more simply, constitute the neutralized form of growth substances in the pollen and the pistil which are inactive as polymers.

6. Structure and Mutability of the Self-incompatibility Locus

The essential information on the structure and mutability of the self-incompatible locus has been obtained or reviewed by SHARMA and BOYES (1961) for the heteromorphic system, by LEWIS (1948, 1951, 1965), LEWIS and CROWE (1953, 1954), PANDEY (1962, 1965, 1967) and DE NETTANCOURT (1972) for the gametophytic species, and by LUNDQVIST (1960, 1964) for the grasses. It is to such reviews that the reader is referred for a complement to the short summary outlined below.

6.1 Structure of the Incompatibility Locus

The incompatibility locus is complex and consists, in heteromorphic species, of linked elements governing stylar length, pollen size, and anther height and regulating stylar and pollen incompatibility. Crossing-over occurs, but very rarely, within the locus.

In the gametophytic system, the S-locus is tripartite (LEWIS, 1965) with:

— A specificity part which individualizes the S-allele and contains the specific genetic information for preventing pollen tube growth in all cases where one of the two specificity parts present in the diploid pistil is also present in the haploid pollen grain.

— A pollen activity part which regulates activity of the specificity part in the pollen.

— A stylar activity part which regulates activity of the specificity part in the style.

6.2 Mutability of the Incompatibility Locus

Evidence has been obtained, in *Lycopersicum*, *Nicotiana*, *Oenothera*, *Petunia*, *Prunus* and *Trifolium*, that each of the three parts of the S-locus is mutable.

Mutations of the activity parts can be induced independently by mutagens and lead to the generation of self-compatible mutants which accept incompatible pollen (stylar-part mutants) or which produce compatible pollen (pollen-part mutants). A considerable amount of work has been carried out to define and to explain the origin and nature of such mutations and the reader is referred, for a general review of the problem, to PANDEY (1965) and DE NETTANCOURT *et al.* (1975).

Mutations of the specificity part, that is to say constructive changes which transform a self-incompatibility allele into another self-incompatibility allele (i.e. $S_1 \rightarrow S_2$), have never been induced by means of mutagenic treatment. A very high rate of spontaneous mutations has, however, been observed to occur in inbred plants (DE NETTANCOURT *et al.*, 1971), which is possibly indicative of mutagenic or switching mechanisms controlled by the general level of homozygosity in the plant. Some evidence has recently been obtained (DEVREUX and RAMULU, in preparation) that the generation of new S-alleles also occurs spontaneously in somatic cells cultured in vitro and used as stem-lines for regenerating entire plants. This finding is particularly interesting because it shows that in vitro culture may be a far more effective mutagen than radiations for inducing constructive mutations in higher plants.

7. Interspecific Incompatibility

It is impossible to define here, in any detail, this wide field of research which has been most extensively reviewed by LEWIS (1958), PANDEY (1962), MARTIN (1961 a, b, 1964), ABDALLA and HERMSEN (1972), HOGENBOOM (1972) and DE NETTANCOURT *et al.* (1974). In summary, it can be said that:

— Interspecific incompatibility is often unilateral and occurs when the species used as staminate parent is self-compatible and the pistillate species is self-incompatible (HARRISON and DARBY, 1955).
— With some variations (see DE NETTANCOURT *et al.*, 1974), the reaction of interspecific incompatibility is morphologically similar to that of self-incompatibility.
— There is, however, considerable controversy as to whether or not interspecific incompatibility is governed by the self-incompatibility locus (for a discussion, see HOGENBOOM, 1972; DE NETTANCOURT *et al.*, 1974).
— KNOX *et al.* (1972) have found that interspecific incompatibility between species of *Populus* depends upon recognition proteins which are located in the wall of the pollen grains. KNOX and his coworkers were able to demonstrate that the interspecific incompatibility barrier could be by-passed if irradiation-killed compatible pollen (mentor pollen), or saline extracts of the wall proteins from unirradiated mentor pollen, were mixed to the incompatible pollen at the time of pollination. The diversity of the pollen wall proteins separated from the mentor pollen was demonstrated by immunodiffusion and immunoelectrophoretic studies, and the detection of specific antigens in the saline leachates was carried out with antisera raised in rabbits. KNOX *et al.* (1972) used the

pollen mixture technique to break down self-incompatibility in the Compositae and concluded, from the significant increase in seed-set which they obtained, that sporophytic self-incompatibility and interspecific incompatibility both depend upon wall proteins in the pollen grains. Observations made on *Lotus* species by GRANT *et al.* (1962), DE NETTANCOURT and GRANT (1963) and MIRI and BUBAR (1966) showed that mentor effects by compatible pollen also lead to a stimulation of seed-set upon selfing in plants with a gametophytic system of self-incompatibility.

8. Incompatibility in vitro

There seem to be at least six different research areas which directly or indirectly relate incompatibility to the use of in vitro techniques. These are:

— The determination of incompatibility substances and of the recognition and rejection phases of the incompatibility reactions (Fig. 1).
— The utilization of incompatibility mechanisms as biological indicators and screening systems (Fig. 1).
— The systematic identification of S-alleles (Fig. 1).
— The modification of incompatibility genotypes in sporophytic systems (Fig. 2).
— The transfer of germplasm between cross-incompatible species (Fig. 2).
— Cryobiology.

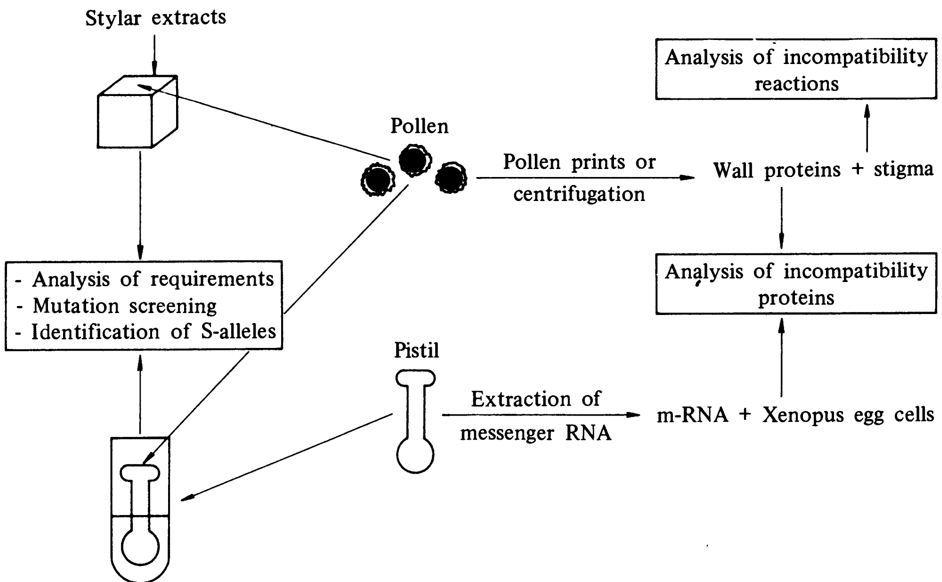

Fig. 1. Examples of the use of in vitro techniques for analyzing the basic features of the incompatibility reaction and for testing the effects of external factors

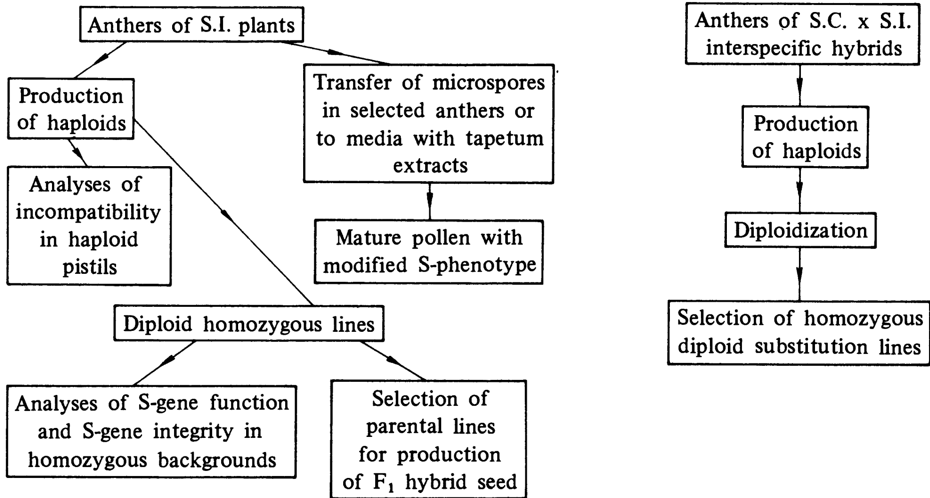

Fig. 2. Examples of the use of anther culture techniques for genetical researches on incompatibility

8.1 Biochemistry of Self- and Cross-incompatibility

8.1.1 The Identification of Incompatibility Substances

The discovery by LEWIS (1952), MÄKINEN and LEWIS (1962) and LEWIS *et al.* (1967) that each S-allele produces a specific antigen which is detectable, in serological tests, at the level of single pollen grains has been fully exploited by KNOX and HESLOP-HARRISON (1970), KNOX and HESLOP-HARRISON (1971) and KNOX *et al.* (1972) for localizing, by immunofluorescence techniques, the incompatibility proteins contained in pollen walls. Now that very small amounts of the wall proteins can be separated by the pollen-print technique (HESLOP-HARRISON *et al.*, 1973; HOWLETT *et al.*, 1973) or by centrifugation (DICKINSON and LEWIS, 1973a, b) for stimulating directly on excised stigmas certain stages of the rejection reaction, it is probable that at least some of the incompatibility components present in pollen grains will soon be purified and identified on the basis of their interactions with analogous substances in excised pistils.

Similarly, since the proper in vitro technique has been established by VAN DER DONK (1974) for inducing *Xenopus* egg cells to synthetize proteins upon injection of messenger RNAs extracted from self- and cross-pollinated styles, it is possible that certain stylar proteins involved in incompatibility reactions will be isolated and characterized in the near future.

8.1.2 Incompatibility Reactions in vitro

Should the above predictions (namely the extraction and identification of incompatibility proteins from pollen and pistil) come true, one could envisage in vitro manipulations for defining, with pollen cultures and with excised pistils, the pre-

cise effects which are inducible in pollen and pistil through the application of incompatibility factors extracted from pollen leachates and incompatible pollen tubes, or produced by *Xenopus* egg cells provided with stylar messenger RNA. Ultimately, the extraction work and the induction procedures could be carried out in sequential orders and in such a manner as to discriminate between recognition and rejection, or to separate distinct steps in the incompatibility reaction. Such a performance would not only permit the testing of various hypotheses on the nature of the reaction, the eventual formation of dimers between pollen and stylar proteins and the basic variations between different systems, it would also allow the establishment, for the botanist, of a unique model for analyzing gene function and gene physiology in vitro.

8.2 Biological Indicators, Screening Systems and Identification of Incompatibility Genotypes

The time however, is not ripe for inducing the various stages of incompatibility reactions in the test tube and using them for testing the effects of substances, treatments, or conditions suspected to interfere with gene integrity or biosynthetic processes such as the formation of S-proteins, the formation of pollen–pistil incompatibility complexes and, eventually, the output of signals for establishing the rejection phase. Yet it is already possible to use certain consequences of the incompatibility reaction (germination failure, growth inhibition, bursting of pollen tubes, callose depositions) for detecting and measuring, in several species of higher plants, incompatibility on excised pistils or on artificial styles. The techniques presently available are such that they allow, for different incompatibility systems, and under in vitro conditions: (1) the scoring of the physiological and genetic effects of treatments designed for preventing incompatibility to occur; (2) the rapid classification of S-alleles and S-genotypes under carefully controlled conditions.

8.2.1 Physiological Effects

The technique of excised pistils cultured in vitro, such as it has been developed, for instance, by KENDALL and TAYLOR (1965) and KENDALL (1968) on *Trifolium pratense*, by HECHT (1960, 1964) and KWACK (1965) for *Oenothera organensis*, or ASCHER and PELOQUIN (1966) and ROSEN (1971) with *Lilium longiflorum*, has proved to be a valuable tool for analyzing the requirements of incompatible pollen tubes and the influence of various treatments upon the rejection process. It is in this manner that BALI and HECHT (1965), ASCHER and PELOQUIN (1966), KENDALL and TAYLOR (1967, 1969), HOPPER *et al.* (1967), KENDALL (1968) were able to demonstrate that high temperatures prevented the self-incompatibility reaction to occur and that the effect was essentially due to an action upon the stylar component of the incompatibility mechanism. Working with *L. longiflorum*, ASCHER and DREWLOW (1971) filled the stylar canal of excised pistils with the stigmatic exudate of other styles and observed that the injected exudate did not

prevent the incompatibility reaction from taking place but, depending upon the time of injection (before or after pollination), increased or decreased the growth of compatible and incompatible tubes. HECHT (1960) devised an ingenious method of grafting by assembling excised stigmas or excised stigmatic lobes of *Oenothera* to excised styles in a solution containing 10% gelatine and 6% lactose; using different combinations of compatible and incompatible stigmas and styles, he was able to show that the incompatibility reaction is stronger in the stigmatic region than in the style. He also established a reliable test system for evaluating, in vitro, the physiological effects of environmental factors, such as temperature, pH, and potassium concentration, on the growth of incompatible pollen tubes. HECHT also detected the effectiveness of warm water, applied 24 h before pollination, for inhibiting self-incompatibility, but other workers have reported that calcium (KWACK, 1965) has an effect on the growth of incompatible pollen tubes in excised pistil and that inhibitors of RNA synthesis prevent the growth of compatible and incompatible pollen tubes (ASCHER, 1971). SARFATTI *et al.* (1974) found, however, that the application of actinomycin D tended to promote the development of incompatible pollen tubes on detached flowers of *Lycopersicum peruvianum.*

It is obvious that researches on the induction and modification of incompatibility reactions in vitro will, from now on, greatly benefit from the refined techniques recently established in *Raphanus* (DICKINSON and LEWIS, 1973a, b), *Iberis* (HESLOP-HARRISON *et al.*, 1974) and *Populus* (KNOX *et al.*, 1972) for detecting incompatibility proteins, extracting them from pollen walls and testing their effects on excised portions of the pistil.

8.2.2 Genetic Effects

In vitro culture techniques are related in two different ways (for a review, see DEVREUX and DE NETTANCOURT, 1974) to the use of the very efficient mutation screening system which is constituted by the tripartite locus of self-incompatibility in gametophytic species. In the first place, anther cultures can give rise, via the production of haploid plants and the constitution, thereafter, of isogenic diploid lines, to the completely homozygous individuals which are needed for testing current hypotheses (DE NETTANCOURT *et al.*, 1971) on the generation of new S-alleles in inbred backgrounds, or for handling genetically uniform populations of microspores and pollen grains. In the second place, in vitro culture techniques can be of great utility for large scale scoring, in excised pistils, of individual pollen grains carrying a self-compatibility mutation at the S-locus. Attempts presently carried out at the Casaccia (DEVREUX *et al.*, in preparation) indicate that one can easily distinguish, in excised stigmas and styles of *O. organensis*, between incompatible pollen tubes and pollen tubes bearing a selfcompatibility mutation induced by radiation treatment in pollen mother cells and young microspores. The screening method, which still needs improvement, definitely opens possibilities for massive in vitro analyses, at the haploid level, of the mutagenic effects of radiations and chemicals on a well-defined genetic locus in multicellular organisms.

8.2.3 Classification of S-alleles and of S-genotypes

In vitro techniques are available for carrying identity tests of the S-alleles and S-genotypes segregating in a population. The most efficient approach defined to date is certainly the Petri dish method elaborated by LUNDQVIST (1961) for self-compatible grasses which allows, 24–30 h after pollination, an accurate scoring of the frequency of emptied (compatible) and still filled (incompatible) pollen grains on excised stigmas. The pollen grains are stained by means of the cotton-blue technique (WATKINS, 1925; HAYMAN, 1956). Similar estimations of compatibility relationships can also be carried out in systems characterized by stylar inhibition, and great use has been made, in this respect of the aniline blue-fluorescence technique (LINSKENS and ESSER, 1957; MARTIN, 1959), which permits to detect, in excised styles, the callosic outlines of incompatible and compatible pollen tubes within the conducting tissue and to record pollen tube lengths or, eventually, the bursting of incompatible tube apices (LANERI and DE NETTANCOURT, 1973). Variability in the growth of compatible and incompatible pollen tubes is such that, with some exceptions like that of *Oenothera* (EMERSON, 1939; DEVREUX *et al.*, in preparation), the scoring of individual pollen mutants is impossible; yet discrimination can easily be made, in most cases, between incompatible, semi-compatible, and compatible pollinations which allows, thereafter, if some tester stocks are available, the identification of the parental genotypes involved in each of the crosses analyzed.

Modifying a technique elaborated by PICARD and DEMARLY (1952), MIRI and BUBAR (1966) have shown that it was possible to carry out identity tests for S-alleles and S-genotypes by examining pollen tube growth on culture media containing extracts of styles having known S-genotypes. MIRI and BUBAR used the method for measuring incompatibility in *Lotus corniculatus* and reported agreement between classifications carried out on such artificial styles and identity tests by means of the conventional fluorescence method. Artificial pistils have also been elaborated by TOMKOVA (1959) for *Nicotiana alata* who observed, in some experiments, that pollen tube growth inhibition occurred when the pollen grains and the stylar extracts had originated from the same plant. Working with *Petunia inflata*, BREWBAKER and MAJUMDAR (1961) considerably improved the method and obtained reproducible results and clear-cut differences between compatible and incompatible pollen tubes grown on culture media with stylar extracts or on excised stylar fragments. There is little doubt that the recent techniques elaborated by DASHEK *et al.* (1972), DICKINSON and LEWIS (1973a, b), HESLOP-HARRISON *et al.* (1974) and VAN DER DONK (1974) will open new perspectives for constructing artificial pistils and for identifying S-alleles in vitro.

8.3 The Modification of Incompatibility Phenotypes in Sporophytic Systems and the Removal of Interspecific Barriers

Since LEWIS and DICKINSON and the team of HESLOP-HARRISON have demonstrated the tapetal origin of the incompatibility proteins localized in the pollen wall of species with a sporophytic system of pre-zygotic rejection, it is possible to

consider, for such species, the modification of the incompatibility phenotype in the pollen. The performance is, in theory, of a simple nature and could be achieved in two ways: (1) by culturing young microspores in artificial media containing the incompatibility proteins which one would like to have incorporated within the exine of the mature pollen grains; (2) by transferring microspores from their original anthers to the anthers of plants having the desired incompatibility genotype.

The techniques involved for bringing cultured microspores to the stage of mature pollen grains or for transferring them from one anther to another are, however, extremely difficult and it is impossible to foresee any routine application of these methods for the near future. Yet researches in the field should certainly be continued, especially in the case of interspecific incompatibility, because one already knows, from the work by KNOX *et al.* (1972) with mentor pollen, that the adjunction of recognition substances from the pollen of one species to the pollen of a second species, allows, at least in *Populus*, the by-passing of important reproductive barriers.

In the meanwhile, attention must of course continue to be given to the arsenal of in vitro techniques available for producing interspecific hybrids by means of embryo culture (see Chap. III.3 of this Vol.). Interspecific incompatibility is usually unilateral and there is generally a possibility of obtaining zygotes or young embryos after crosses between a self-compatible species, used as pistillate parent, and a self-incompatible species employed as pollen source. Post-zygotic barriers often prevent the evolution of such zygotes or embryos which, on appropriate culture media, may evolve into mature plants combining the incompatibility features (intra and interspecific incompatibility) of both parents (see MCGUIRE and RICK, 1954; and DE NETTANCOURT *et al.*, 1974). The technique does not, however, need to be restricted to embryo culture and one must also bear in mind the possible to by-pass incompatibility barriers by means of in vitro fertilization. The performance can be achieved by placing ovules and pollen grains together in the same culture medium (KANTA *et al.*, 1962; MAHESHWARI and KANTA, 1964; RANGASWAMY and SHIVANNA, 1967, 1971a, b; ZENKTELER, 1967, 1970) or by pollinating excised pistils or portions of excised pistils (see, EMSWELLER and UHRING, 1965; DULIEU, 1966, and Chapter III.5).

Last but certainly not the least, one should not forget the potentiality, underlined earlier in this review, of anther and microspore culture techniques for obtaining haploids from self-incompatible plants. The proposition leads to several different fields of application among which one must list:

— The production of haploid plants from interspecific hybrids between self-compatible and self-incompatible species. Such plants should theoretically be genetically different from one another and carry different combinations of paternal and maternal chromosomes. They should, in other words, after diploidization, constitute an extensive collection of substitution lines. The method is at present being tried at the Casaccia Institute where attempts are being made (DEVREUX and LANERI, in progress) to produce, from an interspecific tomato hybrid, haploid and diploid substitution lines with the S-bearing chromosome of *L. peruvianum* in the background genome of *L. esculentum*.

— The use of completely homozygous self-incompatible diploid lines (obtained from doubling the chromosome numbers of haploids) as parental material for the production of F_1 hybrid seed or for analyzing the fate of an incompatibility system suddenly placed in homozygous background.

8.4 Cryobiology and Gene Banks on Artificial Culture Media

It is not the purpose of the present Review to introduce the newly developing field of cryobiology (Chap. VII.3 of this Vol.), nor to discuss the importance of culture techniques for the preservation, as isolated cells, apical meristems, or callus, of valuable plant material. We simply want to point out that self-incompatible plants, if their individual genotypes are to be preserved without any modifications, cannot be stored as seeds which are, by necessity, the result of outcrosses and the carriers of new genetic combinations. Hence, it seems that absolute priority should be given, in gene banks based on the use of in vitro culture methods and on the storing of cultured material at very low temperatures, to the maintenance and preservation of rare genotypes which do not propagate sexually or which lose their integrity from one sexual generation to the next. Self-incompatible plants, and the array of different S-mutants, S-genotypes and background lines which can be selected after various treatments and breeding procedures, obviously belong to this last group.

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Chapter IV

Protoplasts, Somatic Hybridization and Genetic Engineering

1. Protoplast Isolation, Culture and Somatic Hybridization

Y. P. S. Bajaj

1. Introduction

One of the most significant developments in the field of plant tissue culture during recent years has been the isolation, culture and fusion of protoplasts (Cocking, 1972). The techniques are especially important because of their far-reaching implications in studies of plant improvement by cell modification and somatic hybridization (see Bajaj, 1974a). Isolated protoplasts also offer a means of tackling various fundamental and pragmatic research problems in experimental plant biology. This can be realized mainly because of the totipotent nature of plant cells. The literature accumulated so far has revealed that protoplasts in culture can be regenerated into an entire plant; they can be induced to undergo intra- and interspecific fusion to form a somatic hybrid, and also to take up foreign organelles and genetic materials. In addition to general protoplast culture and somatic hybridization studies referred to in this article, protoplasts have been used to investigate various problems in plant physiology (Boulware and Camper, 1972; Bayer, 1973; Hess and Endress, 1973; Kanai and Edwards, 1973; Börner, 1973; Birecka and Miller, 1974; Hall and Cocking, 1974; Hoffmann and Kull, 1974; Gutierrez *et al.*, 1974; Ku *et al.*, 1974; Podbielkowska *et al.*, 1975; Shepard and Totten, 1975), radiobiology (Ohyama *et al.*, 1974; Galun and Raveh, 1975; Howland, 1975; Howland *et al.*, 1975), virology and pathology (Cocking, 1966; Aoki and Takebe, 1969; Hibi and Yora, 1972; Coutts, 1973; Honda *et al.*, 1973; Burgess *et al.*, 1974; Sarkar *et al.*, 1974; Zaitlin and Beachy, 1974; Birecka *et al.*, 1975a, b; Pelcher *et al.*, 1975), cytogenetics (Carlson, 1973a, Chaleff and Carlson, 1974) and cell modification and uptake studies (Davey and Cocking, 1972; Hess, 1973a, b; Hoffmann, 1973; Hoffmann and Hess, 1973; Blaschek *et al.*, 1974; Giles, 1974; Holl *et al.*, 1974).

This fascinating field of research, though still in the formative stages as far as the technology is concerned, has already played an important role in opening up new vistas and has awakened the interest of plant physiologists, pathologists, molecular biologists and cytogeneticists. It is envisaged that in future years protoplasts will be one of the most frequently used research tools for tissue culture studies, and unlimited potential in genetic engineering for plant improvement is foreseen.

This article, in addition to the account on technical aspects of the isolation, culture and fusion of protoplasts is also intended to serve as a general introduction to this chapter on protoplasts.

2. Isolation of Protoplasts

Protoplasts are isolated mainly by two methods (1) mechanical, and (2) enzymatic. The first (KLERCKER, 1892) is used only occasionally (PILET, 1972), but remains historically important, and still has a merit, i.e. the unknown effects of enzymes on protoplasts are eliminated. The enzymatic isolation of protoplasts (COCKING, 1960), on the other hand, offers the advantages (RUESINK, 1971) that (1) the protoplasts can be obtained in large quantities, (2) in contrast to the mechanical methods the cells are not broken, and (3) osmotic shrinkage is much less.

Mechanical and enzymatic methods can also be combined (HARADA, 1973) to retain the merits of both techniques. By this combined method the cells are first separated mechanically, and then enzymatically transformed into protoplasts.

To date, protoplasts have been isolated from almost every part of the plant. Some notable ones are outlined in Table 1, and also listed here: roots (COCKING, 1960; BAWA and TORREY, 1971; KAMEYA and UCHIMIYA, 1972), leaves (TAKEBE *et al.*, 1968; OTSUKI and TAKEBE, 1969; POWER and COCKING, 1970; POTRYKUS and DURAND, 1972; EVANS *et al.*, 1972; DAVEY and SHORT, 1973; SCHASKOLSKAYA *et al.*, 1973; WENZEL, 1973; DAVEY *et al.*, 1974a; KARTHA *et al.*, 1974a, MAEDA and HAGIWARA, 1974; PELCHER *et al.*, 1974; COUTTS and WOOD, 1975; DORION *et al.*, 1975; KOHLENBACH and BOHNKE, 1975; SCHIEDER, 1975a), root nodules (DAVEY *et al.*, 1973) coleoptiles (RUESINK and THIMANN, 1965; HALL and COCKING, 1974), fruit tissues (GREGORY and COCKING, 1965; RAJ and HERR, 1970), pericarp (SKENE, 1974), flower petals (POTRYKUS, 1971a), potato tubers (LORENZINI, 1973), endosperm (MOTOYOSHI, 1972), aleurone layer (TAIZ and JONES, 1971), crown-gall tissue (SCOWCROFT *et al.*, 1973), pollen mother cells and pollen tetrads (BHOJWANI and COCKING, 1972; BAJAJ and COCKING, 1973; BAJAJ, 1974b), pollen grains BAJAJ and DAVEY, 1974; BAJAJ, 1975), and callus cultures (SCHENK and HILDEBRANDT, 1969; ERIKSSON and JONASSON, 1969; HELLMANN and REINERT, 1971; HOLDEN and HILDEBRANDT, 1972; BUTENKO and IVANTSOV, 1973; WALLIN and ERIKSSON, 1973; UCHIMIYA and MURASHIGE, 1974; GOSCH *et al.*, 1975a, b).

2.1 Isolation of Protoplasts from Leaves

The isolation of protoplasts from leaves involves the following four steps: (1) sterilization of leaves; (2) peeling off the epidermis; (3) enzymatic treatment; and finally (4) isolation and cleaning of the protoplasts.

Fully expanded leaves from young plants in a vegetative state (about 10 weeks old in tobacco) are surface-sterilized by dipping them into 70% ethyl alcohol for about a minute, and then treating them for 20–30 min with a 2% solution of sodium hypochlorite. The leaves are then rinsed three times with sterile distilled water to remove traces of sterilant. The subsequent manipulations are carried out under aseptic conditions. Various problems associated with the production of sterile protoplasts have been discussed by WATTS *et al.* (1974).

The lower epidermis from the excised leaves is carefully peeled off (TAKEBE *et al.*, 1968), and the stripped leaves are cut into small pieces. The leaves are rela-

tively easy to peel if the water supply to the plants is limited before excision of the leaves, or if the leaves are allowed to become flaccid after sterilization. Mesophyll protoplasts can be obtained from the peeled leaf segments, while epidermal ones may be obtained from the peeled epidermis (Davey *et al.*, 1974). The protoplasts are generally isolated by the following two methods and the yield can be monitored (Ferrari *et al.*, 1975).

2.1.1 Direct (One-step) Method

The peeled segments are placed with their lower surface downwards in a petri dish containing a filtered-sterile enzyme mixture (0.5% macerozyme + 2% Onozuka cellulase in 13% sorbitol or mannitol at pH 5.4) and incubated overnight (15–18 h) at 25° C (Power and Cocking, 1970). After incubation, the leaf segments are gently teased to liberate the protoplasts. They are then filtered through a fine wire gauze to remove leaf debris, transferred to 13 × 100 mm screw-capped tubes, and centrifuged at 100 g for 1 min. The protoplasts form a pellet while the debris in the supernatant is carefully removed. This process is repeated three times, and the protoplasts are washed with 13% sorbitol solution. For a final cleaning, sorbitol is replaced by 20% sucrose solution, and centrifuged at 200 g for 1 min. Using this procedure the cleaned protoplasts float and the debris settles. The floating protoplasts are gently pipetted out with a Pasteur pipette, and the sample bulked.

2.1.2 Sequential (Two-step) Method

In the protocol outlined below, which is a slight modification of Otsuki and Takebe's (1969) method, cells are first isolated and later transformed into protoplasts.

Step 1. The peeled leaf segments are put in an enzyme mixture A (macerozyme 0.5%, potassium dextran sulphate 0.3% in 13% mannitol at pH 5.8 and vacuum infiltrated (in a desiccator) for about 5 min. They are then transferred to a water bath at 25° C and subjected to slow shaking. After 15 min the enzyme mixture is carefully poured out, replaced by a fresh enzyme mixture, and the leaf segments are incubated for another hour. (At this stage it is advisable to observe a drop of incubating mixture under the microscope to determine if the cells have completely separated.) The mixture is then filtered through a nylon mesh, centrifuged at 100 g for 1 min, and washed three times with 13% mannitol to obtain a pure sample of the isolated cells (Fig. 1A).

Step 2. The cells obtained by step 1 are incubated with enzyme mixture B (2% cellulase in a 13% solution of mannitol at pH 5.4) for about 90 min at 30° C. After incubation they are centrifuged at 100 g for 1 min; the protoplasts form a pellet. The supernatant is removed and the protoplasts (Fig. 1B) are cleaned three times with mannitol. The protoplasts thus isolated can be further purified by floating them on 20–25% sucrose solution (Power and Cocking, 1970). Recently, Kanai and Edwards (1973) have employed a two-phase aqueous system by mixing polyethylene glycol and dextran for the separation of healthy protoplasts. This method can also be used for the separation of different types of cells.

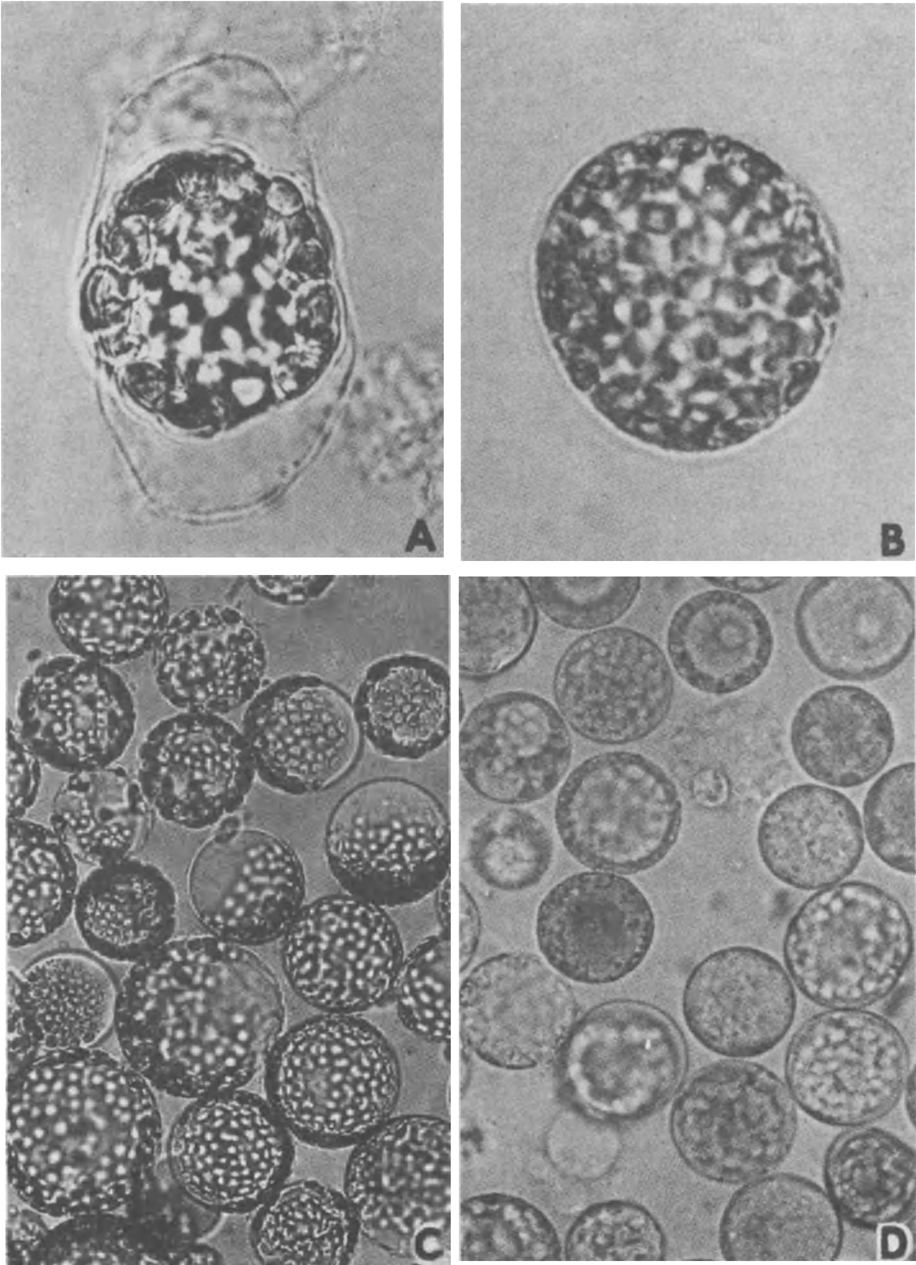


Fig. 1. (A) An isolated mesophyll cell (plasmolyzed) of haploid *Nicotiana Tabacum* cv. White Burley obtained from peeled leaf segments treated with macerozyme (0.5%) in 13% mannitol for one hour. (B, C) Haploid tobacco mesophyll protoplasts obtained from cells incubated with cellulase (2%) solution in mannitol (13%) for 2 h. (D) Leaf protoplasts of rye (*Secale cereale*) obtained from 3-week-old dark-grown seedlings. The sliced leaves have been treated with a mixture of Pectinol Rlo (1.5%) and meicelase (4%) in 15% sorbitol. The protoplasts often show anthocyanin pigmentation

The yield of protoplasts (Fig. 1C, D) obtained by the one-step method is higher, as it contains both the spongy mesophyll and the palisade mesophyll protoplasts, while in the two-step sequential method only palisade mesophyll protoplasts are obtained. In the author's experience with *Nicotiana tabacum* cv. Samsun NN (BAJAJ, HUBER, and VAN KAMMEN, unpublished) the protoplasts isolated by the two-step method are definitely better for culture studies. A long overnight period of incubation (15–18 h) in an enzyme mixture by the one-step method as compared to only 3 h treatment by the two-step method seems to affect the plasmalemma adversely, and thus the subsequent growth behavior of the protoplasts. Another disadvantage of the one-step method is that during isolation the protoplasts tend to undergo spontaneous fusion with protoplast of the adjoining cell through the plasmodesmata (Fig. 7A) and thus protoplasts with more than one nuclei are also obtained.

With minor variation in the technique protoplasts have been obtained from leaves in a large variety of plants (see Table 1). In cereals (EVANS *et al.*, 1972), for instance, where the epidermis is rather difficult to peel, the leaves are cut into narrow longitudinal strips and incubated overnight in an enzyme mixture of meicelase (4%) and Pectinol R1o in 15% sorbitol.

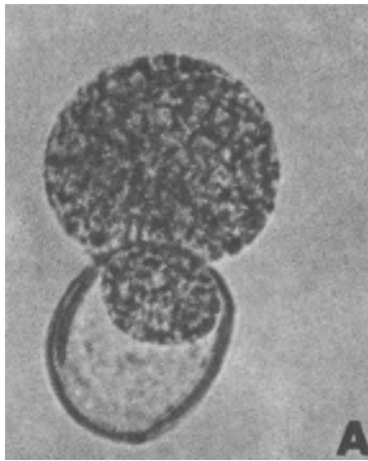
2.2 Isolation of Protoplasts from Callus Cell Suspensions

Actively growing young cell suspensions are ideal material for the isolation of protoplasts in large quantities. Protoplasts can be readily obtained from such cultures by treating the filtered suspensions with 2–4% Onozuka cellulase in 0.6 M mannitol, for 4 to 6 h at 30–33° C in a gently shaking water-bath. With slight modifications, depending on the age and nature of the material, protoplasts have been isolated from tissue cultures of carrot (CHUPEAU and MOREL, 1970; HELLMANN and REINERT, 1971; GRAMBOW *et al.*, 1972; BUTENKO and IVANTSOV, 1973; WALLIN and ERIKSSON, 1973), corn (BAWA and TORREY, 1971; MOTOYOSHI, 1972), *Haplopappus* (ERIKSSON and JONASSON, 1969), rose (PEARCE and COCKING, 1973), tobacco (YAMADA *et al.*, 1972; BUTENKO and IVANTSOV, 1973; UCHIMIYA and MURASHIGE, 1974), soybean (SCHENK and HILDEBRANDT, 1969; KELLER *et al.*, 1970), sugar cane (MARETZKI and NICKELL, 1973) and *Atropa* (GOSCH *et al.*, 1975b).

Older cell cultures have a tendency to form giant cells with thick walls which are difficult and sometimes impossible to degrade by the enzymes. So it is highly desirable that the cell suspensions should be periodically subcultured. Addition of very low concentration (0.1%) of cellulase to cell suspension cultures two days before use, discourages the formation of thick walls. Certain chemicals like colchicine (UMETSU *et al.*, 1975) and chelating agents (EL HINNAWY, 1974), when added to suspension cultures, also tend to prevent the formation of aggregates and consequently yield better cell suspensions.

2.3 Isolation of Pollen Protoplasts

The detailed techniques for the isolation of protoplasts from pollen at various stages of development have been outlined elsewhere (BAJAJ and DAVEY, 1974). It is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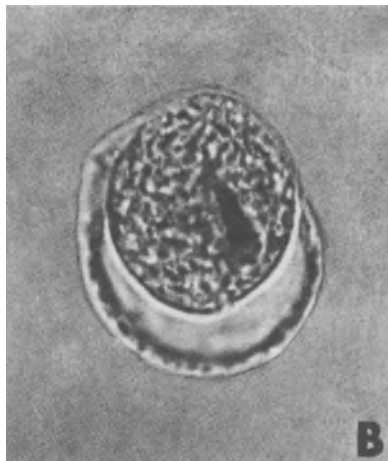
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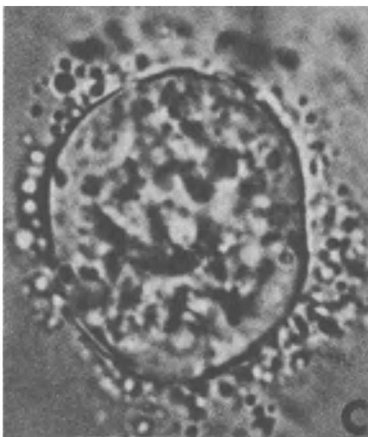
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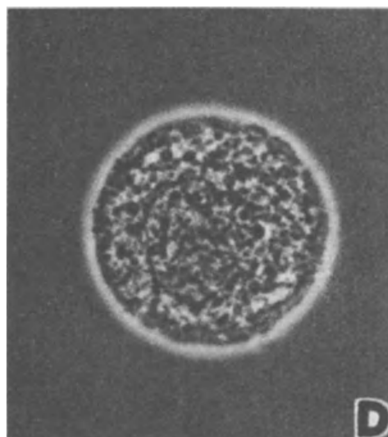
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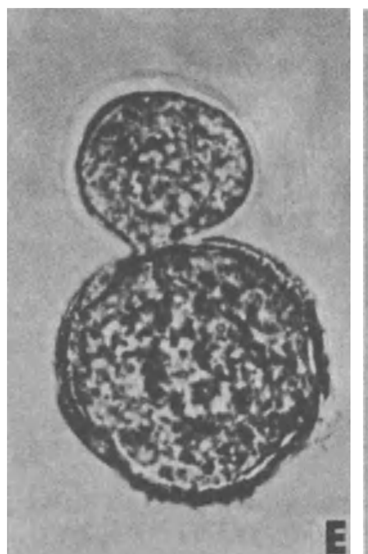
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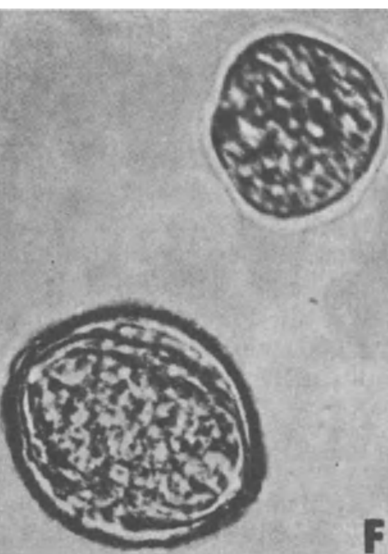
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necessary to reemphasize here, however that pollen protoplasts will have the advantage of being available in large numbers and uniform in ploidy. They could be employed for the study of pollen ontogeny, and perhaps would be more suitable for studies on mutation, somatic hybridization and cell modification. There also exists the possibility of regenerating haploid plants from protoplasts of pollen (BAJAJ, 1974b).

2.3.1 Protoplasts from Maturing Pollen

The major difficulty encountered in the isolation of protoplasts from mature pollen is the complex nature of the pollen wall, which is coated with a remarkably durable and highly resistant substance, sporopollenin. This is a polymer of carotenoid esters (SHAW, 1971), and can normally be dissolved by treatment with strong oxidising solutions—KOH and certain organic bases (SOUTHWORTH, 1974). By enzymatic treatments (BAJAJ and DAVEY, 1974; BAJAJ, 1975) the exine can be partially degraded, and limited quantities of protoplasts have been obtained by the method outlined below.

Pollen, at various stages of development, is squeezed out of the anther, or collected by shaking the mature anthers. The pollen is then sterilized with a 2% (w/v) solution of sodium hypochlorite for 10 min, centrifuged into a pellet (at 300 g for 5 min) and washed twice with distilled water. The chlorine present not only sterilizes the pollen but also bleaches the pollen wall, which permits a better view of the contents of the pollen. The pollen is then incubated with various concentrations and combinations of enzymes. For instance, depending on the stage of development of pollen, up to 25% of *Petunia* pollen treated for 24 h with a mixture of cellulase (2%), macerozyme (1%), helicase (0.5%), rhozyme (1%) and potassium dextran sulphate (0.1% in 15% sucrose) released protoplasts. Replacement with a fresh enzyme mixture after 12 h and frequent agitation during incubation by stirring with a glass rod, sucking the pollen into a Pasteur pipette, or by slight pressure under a glass cover facilitates the release of protoplasts.

By these manipulations protoplasts are released in a variety of ways: (1) Weakening of the germ pore. Since the intine is largely pecto-cellulosic in nature (SITTE, 1953) it is degraded through the germ pore by the mixture of enzyme and the protoplast oozes out (Fig. 2A). (2) Mechanical rupture and sloughing off the exine. The exine, already partially softened by the enzyme, is prone to mechanical handling, which causes breakage or sloughing off. (3) Partial dissolution of the exine. Although sporopollenin is difficult to degrade, the mixture of enzymes is able partially to dissolve the exine (Fig. 2B). Initially the exine appears to swell and disintegrate in the form of globules, which disperse on shaking; but eventually the protoplast is liberated. (Fig. 2C, D). (4) Formation of sub-protoplasts. At higher concentrations of sucrose (20%)

Fig. 2A–F. Various stages in the isolation of protoplasts from maturing pollen. (A) A mature monoporate pollen of *Triticum aestivum* after enzyme treatment; the protoplasts oozes out of the weakend germ pore. (B) Liberation of a protoplast of *Petunia* pollen by partial dissolution of the exine. (C) The exine wall material is being dispersed in the form of globules. (D) A freshly isolated pollen protoplast. (E, F) Liberation of a sub-protoplast from *Petunia* pollen. (After BAJAJ and DAVEY, 1974)

normal germination is inhibited and sub-protoplasts emerge from each of the pores (Fig. 2E, F). In addition, chains of subprotoplasts may be formed from the pollen tube, which are enucleate, and unsuitable for culture purposes.

2.3.2 Protoplasts from Pollen Mother Cells and Pollen Tetrads

When compared to mature pollen, it is relatively easy to obtain protoplasts from pollen mother cells (PMCs) and pollen tetrads (PTs) since they are simple-walled. Each tetrad (Fig. 3A) and PMC is enclosed by a special matrix of callose which is an unbranched β-1:3 glucan. The technique for isolating their protoplasts involves the following steps: (1) One anther from a young flower bud is removed and smeared with acetocarmine to study its stage of development. If the crushed anther contains PTs or PMCs, the bud is retained. (2) With the help of a fine scalpel, anthers are cut into two halves and squeezed gently with a glass spatula. The contents ooze out as milky fluid. (3) The contents are treated with millipore-filtered snail-gut (*Helix pomatia*) enzyme helicase (0.75% in 8% sucrose solution) for 30–45 min and stirred every 15 min. One ml of the enzyme is used per anther. (4) After incubation, the enzyme mixture is gently pipetted out and the protoplasts washed a couple of times with sucrose solution and allowed to settle.

The enzyme mixture is rich in β-1:3 glucanase activity, and is capable of digesting both the callose and to some extent cellulosic material (PINTO DA SILVA, 1969). The callose around the PTs and PMCs begins to dissolve (Fig. 3B) in 10 min and almost 100% of the protoplasts are released in 45 min (Fig. 3C). Immediately following their release from their walls, the protoplasts show a considerable increase in size. They are spherical and densely cytoplasmic with a prominent centrally located nucleus, and the plasmalemma is fairly clean and devoid of any overlying wall material (Fig. 3D).

3. Protoplast Culture and Regeneration of Plants

Plant cells are totipotent and have the capacity to unfold their morphogenetic potential to develop into whole plants (BRAUN, 1959) and trees (WINTON, 1970). Likewise, isolated protoplasts (in culture) regenerate a cell wall around themselves to reconstitute a cell and undergo repeated divisions to form a callus. By manipulation of the nutritional and physiological conditions this cultured tissue may be induced to regenerate into an intact plant. Protoplasts also have the remarkable property of taking up small and large molecules, viruses, bacteria, chloroplasts, DNA and whole nuclei. This suggests a method by which "transformed" plants may be obtained. In the following section an account of the techniques for the protoplasts culture is given and also a description of the various developmental processes which lead to the regeneration of intact plants.

Protoplasts can be suspended in a liquid medium in Erlenmeyer flasks without shaking and can be cultured in small quantities in hanging drops (BAWA and TORREY, 1971), or in microchambers (VASIL and VASIL, 1973). The microchamber is prepared by placing two 22 mm^2 cover slips about 18 mm apart on a drop of

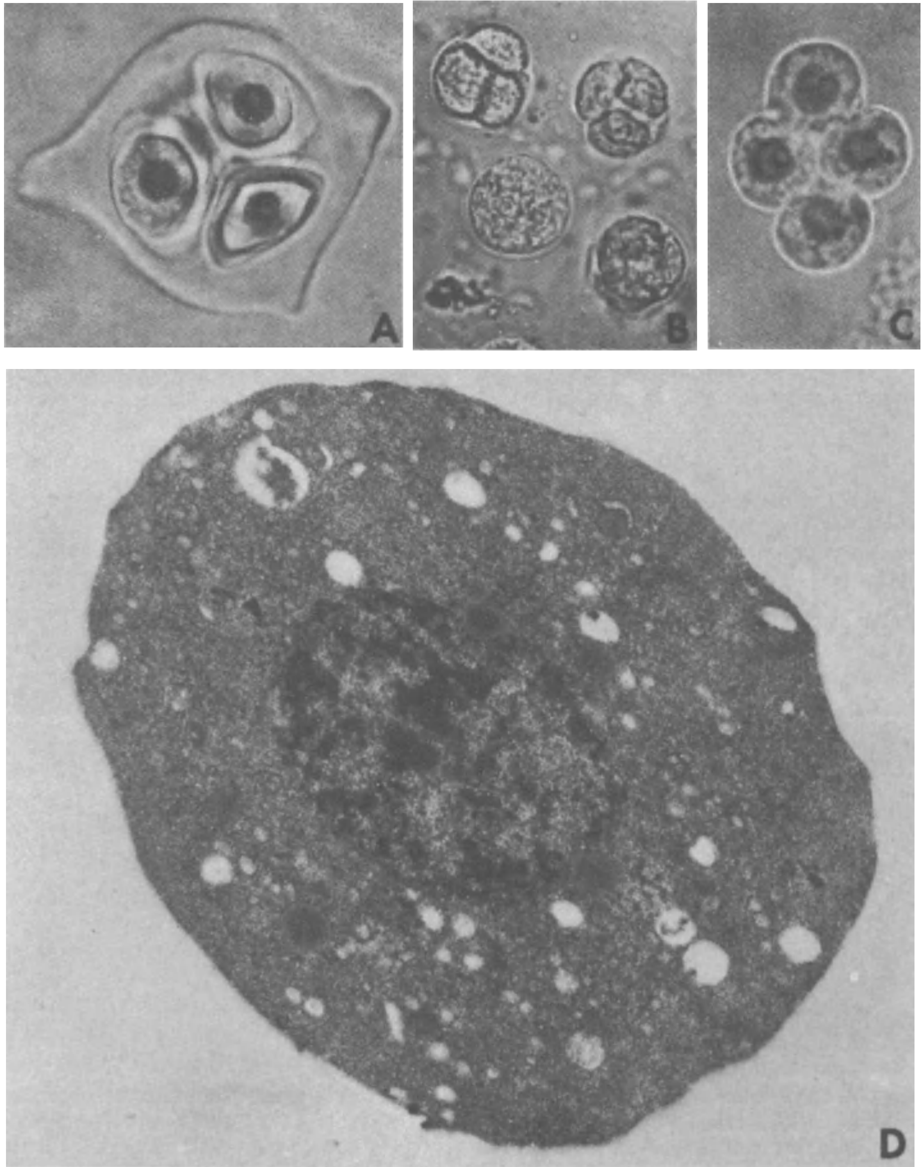

Fig. 3A–D. Isolation of protoplasts from pollen tetrads. (A) Pollen tetrad of *Nicotiana tabacum* cv. White Burley enclosed by a thick callose wall. (B,C) Same, after 10 and 20 min incubation with helicase (0.75%) in 8% sucrose; note the dissolution of callose wall and the liberation of protoplasts. (D) Electron micrograph of a pollen tetrad protoplast released from the callose wall. The protoplast is highly cytoplasmic with a centrally located nucleus: note the clean plasmalemma devoid of wall material. (After BAJAJ *et al.*, 1975a)

heavy mineral oil on a slide. A drop of medium containing protoplasts is placed in between the two cover slips. A third cover slip is then gently placed on the medium and finally sealed with mineral oil. Using these methods protoplasts can undergo a limited number of divisions and may eventually be transferred to an agar medium for further growth to proceed.

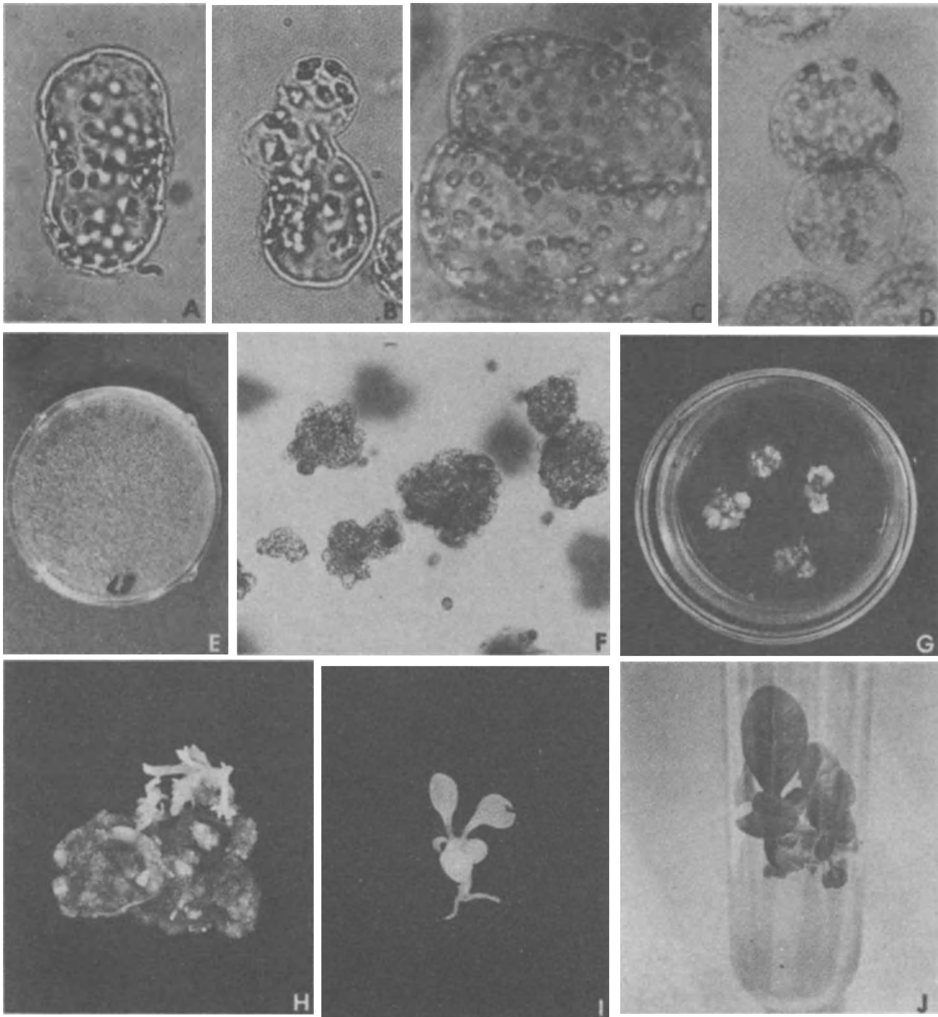

Fig. 4A–J. Various stages in the culture and regeneration of plants from haploid *Nicotiana tabacum* cv. White Burley mesophyll protoplasts. (A, B) Protoplasts 2 and 5 days after plating on a modified Murashige—Skoog's medium (NAGATA and TAKEBE, 1971) containing extra 13% mannitol and 1% sucrose; note the elongation in (A) and budding in (B). (C, D) 5- and 7-days-old dividing protoplasts, the chloroplasts are becoming less dense. (E) Protoplasts 21 days after plating; note the formation of small colonies of cells. (F) Cell clumps and colonies from 4-week-old culture. (G) 8-week-old masses of callus tissue. (H) 12-week-old callus transferred to a differentiation inducing medium (MS + IAA 2 mg/l + kinetin 2 mg/l) showing regeneration of shoots. (I, J) Plantlets obtained from isolated protoplasts; total time after culture 15 and 20 weeks respectively. (After BAJAJ, 1972)

Perhaps the best method for the culture of protoplasts is by plating them in agar-solidified medium—a technique first employed to grow isolated callus cells of tobacco and bean (BERGMANN, 1960), and since then modified (NAGATA and TAKEBE, 1971) for the culture of isolated protoplasts. By this method a large number of cells/protoplasts can be handled conveniently, observed under the

microscope, and plating efficiency easily determined. Two ml aliquots of protoplasts (density about 10^5/ml) suspended in the liquid culture medium are poured into small plastic petri dishes, and mixed gently with an equal volume of the same medium containing 1.2% agar. At the time of mixing, particular care should be taken to ensure that the temperature of the medium is not higher than 45° C. It is advisable to seal the petri dishes with Parafilm M to prevent desiccation. The dishes are then incubated in an inverted position at 25–28° C. It is pertinent to point out here that protoplast density and light intensity are critical to maximum plating efficiency, and therefore these conditions must be controlled accordingly. In general, protoplast density within the range of 5×10^4 to 10^5/ml seems to be suitable. Regarding light intensity, it has been reported (ENZMANN-BECKER, 1973) that the plating efficiency of tobacco protoplasts may be considerably enhanced if they are incubated for the first two days at low light intensity (300 lux) and then transferred to a high light regime (3000 lux).

Normally, for the induction of division in the protoplasts, they have to be plated at final densities higher than 10^4/ml of the medium. However, at such densities there is often coalescence of adjacent cells and somehow it is difficult to pick up a colony of single cell origin; chimeral tissues are also formed. RAVEH *et al.* (1973) adopted a novel approach using non-dividing X-irradiated protoplasts as a feeder layer to support the division of viable protoplasts plated at a density as low as 10^2/ml. This technique would be especially advantageous for the selection of colonies formed from single mutant cells.

3.1 Cell Wall Regeneration

Protoplasts in culture start to regenerate a wall within a few hours, and may take two to several days to complete it (POJNAR *et al.*, 1967; HORINE and RUESINK, 1972). The newly synthesized wall can be demonstrated either by plasmolyzing the reconstituted cell, or by staining it with Calcofluor white fluorescent stain (0.1%). However, more important are the electron microscopic, surface replica and freeze etching studies. The nature of the new wall varies a great deal and extensive electron microscopic work (see COCKING, 1966; WILLISON, 1973; TAKEBE and OTSUKI, 1973; FOWKE *et al.*, 1973; DAVEY *et al.*, 1974; BAJAJ *et al.*, 1975a) on protoplast wall regeneration reveals that wall materials are progressively deposited at the surface of the plasmalemma within a few hours of the start of culture. In some cases, a multilamellar wall material deposition precedes the formation of a cellulosic wall. The cellulose is deposited either between the plasmalemma and the multilamellar wall material, or directly on the plasmalemma. The nature of the biosynthesis of the wall (ALBERSHEIM, 1974) varies considerably depending on the plant material and the system under investigation. Where protoplasts are cultured at high density, i.e. almost touching one another, there is coalescence and common walls are regenerated resulting in the formation of cell aggregates (COCKING, 1970) or "chimerical tissue". In the cultures of protoplasts from tobacco pollen tetrads (BAJAJ *et al.*, 1975a), electron microscopic studies revealed that at the point of contact and adhesion of the plasmalemma, the wall materials secreted by the protoplasts merged with one another to form a common wall giving rise to a "pollen tissue".

3.2 Growth and Division

Regeneration of a wall is not a prerequisite for nuclear division, as has been observed with carrot protoplasts (REINERT and HELLMANN, 1971)—they undergo one or several nuclear divisions to become multinucleate without undergoing cytokinesis. This could possibly be due to cell wall removal which results in an imbalance between mitosis and cytokinesis (ERIKSSON and JONASSON, 1969). However, a more logical explanation seems the use of rather higher concentrations of impure enzymes and long-period incubations which damage the plasmalemma rendering it incapable of performing its normal function—the regeneration of the wall.

Likewise, *Haplopappus* (ERIKSSON and JOHASSON, 1969) and *Convolvulus* (BAWA and TORREY, 1971) protoplasts, although regenerating walls, underwent only one or two nuclear divisions with no cytokinesis being observed. In other systems there is only limited protoplast division to form two cells, e.g. isolated pollen tetrad and pollen mother protoplasts (BAJAJ, 1974b) undergo one or two divisions. After wall regeneration, the reconstituted cell shows a considerable increase in size, and the first division is observed after 3–5 days. For example, in tobacco after wall regeneration, the protoplasts undergo an increase in size, elongate (Fig. 4A, B) and the chloroplasts become organized. The cells start to divide (Fig. 4C, D) after three days and show both equal as well as unequal divisions. The chloroplasts lose their green color, become yellowish and are scantily distributed. The second division is observed within a week and after another week aggregates of cells are formed (Fig. 4E, F). After three weeks light green colonies are visible, the cells vacuolate with little or no sign of the presence of chloroplasts, and the colonies attaining a size of approx. 1 mm in about six weeks.

Once small colonies are formed, their further growth is slowed down or inhibited altogether if they are allowed to remain on the original high osmotic medium. The colonies, therefore, should be picked up and transferred to a mannitol-free medium. Sometimes, aggregates of cells and small colonies are difficult to pick up, or they get damaged by rough handling with the forceps. In such cases it is advisable to cut out small pieces of agar containing the colonies, and transfer them onto the top of another medium on which masses of callus are formed (Fig. 4G).

3.3 Regeneration of Plants

At present the induction of embryogenesis or the regeneration of complete plants is restricted to only a few species, i.e. *Asparagus officinalis* (BUI-DANG-HA and MACKENZIE, 1973), *Atropa belladonna* (GOSCH *et al.*, 1975b), *Brassica napus* (KARTHA *et al.*, 1974b), *Citrus sinensis* (VARDI *et al.*, 1975), *Datura innoxia* (SCHIEDER, 1975a), *Daucus carota* (KAMEYA and UCHIMIYA, 1972; GRAMBOW *et al.*, 1972; GOSCH *et al.*, 1975a), *Nicotiana* (NAGATA and TAKEBE, 1971; NITSCH and OHYAMA, 1971; TAKEBE *et al.*, 1971; BAJAJ, 1972; CARLSON *et al.*, 1972; DAVEY *et al.*, 1974b; GLEBA *et al.*, 1974; RAVEH and GALUN, 1975), *Petunia* (DURAND *et al.*, 1973; FREARSON *et al.*, 1973; DONN *et al.*, 1973; BINDING, 1974a;

HAYWARD and POWER, 1975), *Ranunculus scleratus* (DORION *et al.*, 1975). While in *Pharbitis nil* (MESSERSCHMIDT, 1974) only roots, and in *Cucumis sativus* (COUTTS and WOOD, 1975), *Hyoscyamus niger* (KOHLENBACH and BOHNKE, 1975) *Parthenocissus tricuspidata* (SCOWCROFT *et al.*, 1973), *Phaseolus vulgaris* (PELCHER *et al.*, 1974), *Pisum sativum* (CONSTABEL *et al.*, 1973), *Vigna sinensis* (DAVEY *et al.*, 1974), *Vitis vinifera* (SKENE, 1974) and *Saccharum officinarum* (MARETZKI and NICKELL, 1973) calli have been induced (for details see Table 1). The first step for the regeneration of plants involves the transference of a callus mass to a medium capable of inducing differentiation. The general principles applicable to tissue cultures hold good for the calli obtained from protoplasts. The removal of cell wall does not in any way impair the embryogenic potential, and the calli obtained from protoplasts of carrot and *Atropa* (GOSCH *et al.*, 1975a, b), like those from the original callus stock, undergo embryogenesis on an auxin-free medium. Also, an appropriate balance of auxin and cytokinins induces differentiation in most of the system investigated so far—tobacco callus obtained from protoplasts is induced to differentiate on naphthaleneacetic acid and benzylamino purine (NAGATA and TAKEBE, 1971).

In tobacco, small cell colonies or calli differentiate shoots within 3–4 weeks when transfered to a medium containing IAA (4 mg/l) and cytokinin (2 mg/l) (Fig. 4H), and occasionally roots are also formed (Fig. 4I). However, in cases where roots are not formed, they can be induced by transferring the shoots to a basic White's medium (WHITE, 1963). The plants (Fig. 4J) are subsequently transferred to pots. The time from the incubation of protoplasts to the regeneration of an entire plant varies between 7–10 weeks. The isolation, culture and regeneration of plants from protoplasts is diagrammatically represented in Figure 5.

Although the number of reports so far available on the regeneration of plants from isolated protoplasts is limited, the extension of these techniques to other plants presumably depends on the finding of suitable media and conditions. With this sudden outburst of interest, and the rapid increase in the number of workers in protoplast culture work, more reports are expected shortly.

3.4 Genetic Engineering through Protoplast Culture

Modification of cells by genetic engineering renders genes manipulable in ways which were not possible before. This opens up new vistas and a host of scientifically interesting prospects for plant improvement (see HEYN *et al.*, 1974; Chap. IV.3). In this connection plant protoplasts have the remarkable property of being able to take up macromolecules and particles such as ferritin, polystrene, latex, proteins, DNA, viruses, bacteria and even whole nuclei and isolated chloroplasts. This property of protoplasts can be utilized for the incorporation of desirable information into plants.

3.4.1 Virus Uptake

Studies on the mechanism of infection, and host-parasite relationships at the single cell level have been hampered in the past for a number of reasons. Though callus cultures have been employed for infection studies with tobacco mosaic

Table 1. Plant species in which protoplasts have been successfully cultured

Plant species	Origin of protoplasts	Enzyme mixture	Culture medium	Growth response	Reference
Asparagus officinalis L. cv. Marché de Malines	Cladode's (leaf) cells	Macerozyme (1%) + Cellulase Onozuka SS (3%) in 0.9 M mannitol	Mod. MS + NAA (1 mg/l) + zeatin (0.3 mg/l)	Roots and Shoots	BUI-DANG-HA and MACKENZIE (1973)
Atropa belladonna cv. lutea Döll	Stem-callus	Cellulase R 10 (1.5%) + Macerozyme R 10 (0.75%) in 0.5 M sorbitol	Mod. MS + NAA (2mg/l) + kinetin (0.5 mg/l)	Embryos and Plants	GOSCH *et al.* (1975b)
Brassica napus L. (rape) cv. Zephyr	Mesophyll cells	Cellulase Onozuka P 1500 (0.5%) + Rhozyme HP 1500 (0.5%) + Driselase (0.5%) + Hemicellulase (0.5%) in sorbitol (4.5%) + mannitol (4.5%)	B_5 + NAA (10^{-6} M) + BA (10^{-6}M)	Plants	KARTHA *et al.* (1974b)
Citrus sinensis (Shamouti orange)	Ovular callus	Cellulase (1%) + Pectinase (1%) + PDS (0.3%) in 0.14 M sucrose + 0.28 M mannitol + 0.28 M sorbitol	MURASHIGE and TUCKER's (1969) basal medium	Embryos	VARDI *et al.* (1975)
Cucumis sativus L. (cucumber) cv. Ashley cv. China	Mesophyll cells	Preplasmolysis in a salt medium, and Cellulase (0.3%) + Pectinase (0.4%) + PDS (0.5%) in 11% mannitol	HARADA's medium (1973)	Cell clusters	COUTTS and WOOD (1975)
Datura innoxia	Mesophyll cells	Macerozyme (1%) + Cellulase Onozuka (3%) in 0.5 M mannitol	DURAND *et al.* (1973) + NAA (0.5 mg/l) + BAP (0.4 mg/l)	Plants	SCHIEDER (1975a)

Table 1 (continued)

Plant species	Origin of protoplasts	Enzyme mixture	Culture medium	Growth response	Reference
Daucus carota (carrot) cv. Kokubun Senke, Daiche, Ninzin	Root-cells	Pectinase (0.1%) + Cellulase (5%) in KCl (3.5%) + CaCl (0.5%)	Basal medium + NAA or 2,4-D (0.1 mg/l) + coconut milk (1%).	Embryos	KAMEYA and UCHIMIYA (1972)
cv. Royal Chantenay	Root and Petiole callus	Cellulase Onozuka P 5000 (0.5%) + Rhozyme HP 150 (0.25%) in 0.56 M mannitol	B_5 + 2,4-D (0.1 mg/l)	Plants	GRAMBOW *et al.* (1972)
cv. Royal, Chantenay	Root callus	Cellulase Onozuka 4S (5%) + Macerozyme (1%) + 0.05 M citrate buffer in 0.4 M sorbitol	Basal medium + 2,4-D (0.1 mg/l) + 6-(3-methyl-2-butten-1-ylamino)-purine (0.1 mg/l)	Callus	WALLIN and ERIKSSON (1973)
cv. Rote Riesen	Root-callus	Desalted Cellulase (1.5%) in 0.6 M sorbitol	Mod. MS or B_5	Embryos	GOSCH *et al.* (1975a)
Hyoscyamus niger L. cv. *annuus* Sims	Mesophyll cells	Pectinol (0.02%) + Cellulase Onozuka R 10 in KCl (2.5%) + $MgSO_4$ (1%)	DURAND *et al.* (1973) with 0.6 M mannitol	Callus	KOHLENBACH and BOHNKE (1975)
Nicotiana tabacum cv. Xanthi (Diploid)	Mesophyll cells	Macerozyme (0.5%) + Cellulase Onozuka (2%) in 0.7 M mannitol	NAGATA and TAKEBE (1971) + NAA (3 mg/l) + 6-BAP (1 mg/l)	Plants	NAGATA and TAKEBE (1971)
cv. Xanthi	Mesophyll cells	Same	KOHLENBACH'S medium (1966) + NAA (3 mg/l)	Plants	TAKEBE *et al.* (1971)
cv. Red-Flowered (Haploid)	Mesophyll cells	Macerozyme (0.5%) + Cellulase Onozuka (2%) in 0.7 M mannitol	Basal medium + 2,4-D (1 mg/l) + 6-BAP (1 mg/l)	Plants	NITSCH and OHYAMA (1971)
cv. White Burley (Haploid)	Mesophyll cells	Macerozyme (0.5%) + Cellulase Onozuka (2%) in 0.8 M mannitol	NAGATA and TAKEBE (1971) + IAA (3 mg/l) + kinetin (1 mg/l)	Plants	BAJAJ (1972)

Table 1 (continued)

Plant species	Origin of protoplasts	Enzyme mixture	Culture medium	Growth response	Reference
cv. Xanthi (Haploid and Diploid)	Mesophyll cells	Cellulase (1%)+ Macerozyme (1%)+ Driselase (1%)+ PDS (0.3%) in 0.7 M mannitol	Mod. NAGATA and TAKEBE'S medium (see GALUN and RAVEH, 1975)	Plants	RAVEH and GALUN (1975)
cv. Xanthi (Diploid)	Epidermal cells	Macerozyne (0.4%)+ Meicelase (4%) in 0.7 M mannitol	Same	Plants	DAVEY *et al.* (1974b)
cv. Samsun NN	Mesophyll cells	Macerozyme (0.5%)+ Cellulase Onozuka (2%) in 0.7 M mannitol	Same	Callus	BAJAJ, HUBER and VAN KAMMEN (unpublished)
cv. Xanthi and Samsun (Diploid and Haploid)	Mesophyll cells	Macerozyme R 10 (0.25%) + Cellulase Onozuka (2.5%) in 0.4 M mannitol	NAGATA and TAKEBE (1971)+NAA (3 mg/l) + kinetin (1 mg/l)	Plants	GLEBA *et al.* (1974)
cv. Vi $-$ A_1 (virescent) Sl_1Sl_2 (sublethal)	Mesophyll cells	Macerozyme (0.5%)+ Cellulase Onozuka (2%) in 0.7 M mannitol	KOHLENBACH'S medium (1966)+NAA (3 mg/l)	Somatic Hybrids	MELCHERS and LABIB (1974)
Nicotiana glauca × *N. langsdorffii*	Mesophyll cells	Macerozyme (0.4%)+ Cellulase Onozuka SS (4%) in 0.6 M sucrose	NAGATA and TAKEBE+NAA (3 mg/l) +6-BAP (1 mg/l)	Somatic Hybrids	CARLSON *et al.* (1972)
Parthenocissus tricuspidata	Crown-gall	Macerozyme R 10 (0.01%)+ Cellulase Onozuka R 10 (2%) in 13% mannitol	NAGATA and TAKEBE (1971)+NAA (0.1 mg/l)+ kinetin (0.2 mg/l)	Callus	SCOWCROFT *et al.* (1973)
Petunia hybrida cv. Cyanidintyp	Mesophyll cells	Pectinase (2%)+Cellulase Onozuka SS (2%) in 0.4 M mannitol	NITSCH medium (1969) +Reibsaft (2%)	Plants	DURAND *et al.* (1973)

Table 1 (continued)

Plant species	Origin of protoplasts	Enzyme mixture	Culture medium	Growth response	Reference
cv. Blue Dandy, Gypsy, Dream Girl, Red Cap	Mesophyll cells	Macerozyne (0.4%) + Cellulase Onozuka P 1500 (1.2%) in 13% mannitol	Basal medium + 2,4-D (0.5 mg/l) + NAA (1.5 mg/l) + 6-BAP (1 mg/l)	Plants	FREARSON *et al.* (1973)
strain H 229 B 1100d	Mesophyll cells	Macerozyme (1%) + Onozuka 4S (2—3%) in 0.5 M mannitol	Modified DURAND *et al.* (1973) + extra $CaCl_2$ (5 mMol) + $MgSO_4$ (4 mMol)	Plants	BINDING (1974a)
Petunia parodii	Mesophyll cells	Meicellase (3%) + Macerozyme (0.3%) in 13% mannitol	Basal medium + 2,4-D (0.5 mg/l) + NAA (1.5 mg/l) + 6-BAP (1 mg/l)	Plants	HAYWARD and POWER (1975)
Pisum sativum L. (Pea) cv. Century	Mesophyll cells	Purified Cellulase (1%) + purified Hemicellulase (1%) + purified Pectinase (0.5%) in 0.55 M sorbitol and B 5 mineral salts	B5 + NAA (1 mg/l) + kinetin (2 mg/l)	Callus	CONSTABEL *et al.* (1973)
Pharbitis nil cv. Violet	Cotyledon's mesophyll cells	Pectinase (2%) + Cellulase Onozuka SS (3%) in 0.3 M mannitol	NAGATA and TAKEBE (1971) + NAA (1.5 mg/l) + 6-BA (0.5 mg/l)	Callus and Roots	MESSERSCHMIDT (1974)
Phaseolus vulgaris (Bean) cv. Pinto	Mesophyll cells	Pectinase (0.25%) + Cellulase (0.25%) in 0.3 M mannitol	B_5 + 2,4-D (1 mg/l) + kinetin (2 mg/l) + casein hydrolysate (1000 mg/l)	Callus	Pelcher *et al.* (1974)
Ranunculus sceleratus L.	Mesophyll cells	Cellulase Onozuka R 10 (0.1%) + Driselase (0.05%) + Macerozyme R 10 (0.02%) in 10% mannitol	Basal medium + NAA (3 mg/l) + BAP (1 mg/l)	Plants	Dorion *et al.* (1975)

Table 1 (continued)

Plant species	Origin of protoplasts	Enzyme mixture	Culture medium	Growth response	Reference
Saccharum officinarum (sugar cane)	Callus	Cellulase Onozuka 4S (5%)+ Glusulase (1.5%)+ sorbitol 0.4 M+ glucose 5.5 mM	Mod. WHITE'S medium+ yeast extract	Callus	MARETZKI and NICKELL (1973)
Vigna sinensis cv. Black eye	Mesophyll cells	Meicellase (4%)+ Macerozyme (0.4%) in 0.7 M mannitol	NAGATA and TAKEBE'S medium (1971)	Callus and Roots	DAVEY *et al.* (1974a)
Vitis vinifera (grape vine) cv. Sultana	Pericarp callus	Cellulase Onozuka SS (2%)+ Macerozyme (1%) in 0.1 M $CaCl_2$ +0.14 M KCl	B_5+casein hydrolysate (2000 mg/l)+ kinetin (0.2 mg/l)+ NAA (0.1 mg/l)	Callus	SKENE (1974)

virus and its RNA (see ZAITLIN and BEACHY, 1974), the process was not synchronous, and because of the presence of the cell wall the extent of infection was largely restricted to cells with broken or damaged walls. However, when the wall is enzymatically removed, the protoplasts can be infected synchronously.

COCKING (1966) observed the uptake of tobacco mosaic virus by tomato fruit protoplasts and studied the initial stages of infection by electron microscope. Since then it has been reported that protoplasts from other plants can be infected by TMV (HIBI *et al.*, 1968; AOKI and TAKEBE, 1969; COCKING and POJNAR, 1969; OTSUKI *et al.*, 1972; HIBI and YORA, 1972; COUTTS, 1973), cowpea chlorotic mottle virus (MOTOYOSHI *et al.*, 1973), potato virus X and cucumber mosaic virus (OTSUKI and TAKEBE, 1973). These studies have demonstrated that TMV/RNA is taken up by pinocytosis through the plasmalemma, and that the infection is stimulated by poly-L-orinithine. The virus enters the cytoplasm of the protoplast and multiplies. The intracellular amount of virus is almost equal to that formed in the cells of infected growing plants. Infection is synchronous and 60–80% of the cells have been infected; about the same amount of virus is produced in 2–3 days in protoplasts as in ten days in a similar number of cells of an intact growing plant (COUTTS, 1973). Protoplasts can also be infected by more than one virus at the same time (TAKEBE and OTSUKI, 1974). Studies such as these might give insight into (1) the effect of one virus on the other in the same host, (2) the mechanism of infection, and (3) nuclear protein replication.

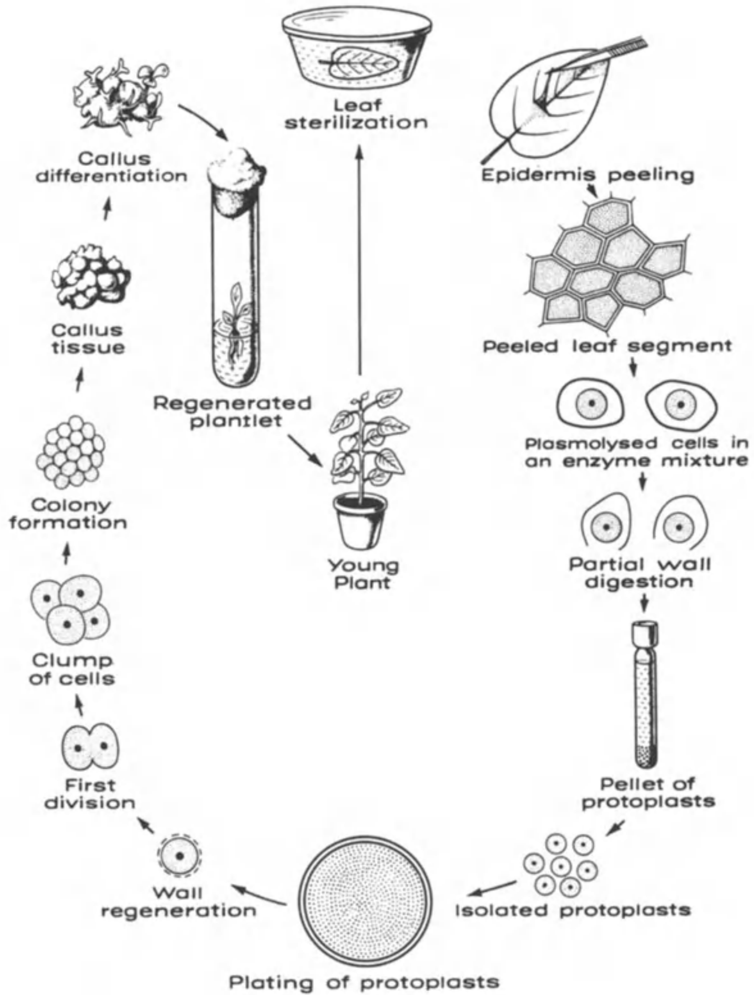

Fig. 5. Schematic sequence for the isolation, culture and regeneration of plants from leaf protoplasts. (After Bajaj, 1974a)

3.4.2 Baterial Uptake and Nitrogen Fixation

Symbiotic nitrogen fixing bacterium *Rhizobium* can be introduced into legume protoplasts during enzymatic digestion of the cell wall (Davey and Cocking, 1972). This uptake occurs by invaginations of the plasmalemma during plasmolysis. Legume root-nodule protoplasts containing packets of bacteria have also been successfully isolated (Davey *et al.*, 1973). These nodule protoplasts could be fused with nonlegume protoplasts and such investigations could result in new endosymbiotic relationships. The introduction of free nitrogen fixing bacteria, like *Azotobacter*, and the blue-green algae into nonlegumes are possibilities for the future. Information for nitrogen fixation could also be transferred to the

protoplasts by plasmids, which are much smaller than the bacteria. The incorporation of nitrogen fixing genes (nif genes) into nonlegumes (see CHILD, 1975; SCOWCROFT and GIBSON, 1975), especially cereals which are already photosynthetically efficient would render these plants self sufficient for increased protein synthesis. This is another area where geneticists and agronomists could combine their efforts.

3.4.3 Incorporation of Exogenous DNA

Transformation in bacteria by exogenous DNA (OSWALD *et al.*, 1944) is an established fact. However, the information accumulated during the last decade (LEDOUX, 1965; LEDOUX *et al.*, 1971a, b; LEDOUX and HUART, 1974; HESS, 1970a, 1972a; see Chap. IV. 3) suggests that similar techniques might be used with higher plant cells. Despite some scepticism (HOTTA and STERN, 1971), these manipulations may yet prove very important. Exogenous DNA can be taken up by higher plant cells/protoplasts (ANKER and STROUN, 1968; BENDICH and FILNER, 1971; OHYAMA *et al.*, 1972; HOFFMANN and HESS, 1973; HOLL *et al.*, 1974). Recently, the work of DOY *et al.* (1972, 1973a, b) has attracted much attention. They claimed to have successfully transformed haploid tomato and *Arabidopsis* callus cultures by *lambda* phage. The fact that these "transformed" higher plant cells can grow on a medium containing galactose and lactose as the exclusive source of carbon is regarded by these workers to be sufficient proof that phage DNA is functional. These authors coined a new term "Transgenosis" for this type of transformation in a higher plant system. Similar observations have been reported by JOHNSON *et al.* (1973), who incubated sycamore *(Acer pseudoplatanus)* cells in a bacteriophage suspension for two days, and then cultured these treated cells on a lactose medium. The control cells died, but the cells incubated with λplac 5 continued to grow. They conculuded that the *lac* genes from the bacterium are expressed and this leads to the formation of a bacterial β-galactosidase which confers the capacity to assimilate lactose.

These studies on transgenosis, although lacking convincing evidence and prone to criticism, would provide new gene pools if perfected. It is undeniable that transfer of DNA from a disease-resistant cell into a susceptible cell/protoplast would be a major advancement in plant breeding and pathology.

3.4.4 Transplantation of Nuclei

Organelles and other small particles have been observed to enter protoplasts; however, it is fascinating that organelles as large as nuclei can be introduced through the plasmalemma into the protoplast without causing it to burst (POTRYKUS and HOFFMANN, 1973). Both, intra- and interspecific nuclear transplantations have been observed in *Petunia hybrida*, *Nicotiana tabacum* and *Zea mays*. Whether these implanted nuclei retain their normal function, or are degraded during culture remains to be seen. Certainly it opens up new avenues for cytogeneticists to study nuclear-cytoplasmic interactions, more so if fertile plants could be regenerated from such protoplasts.

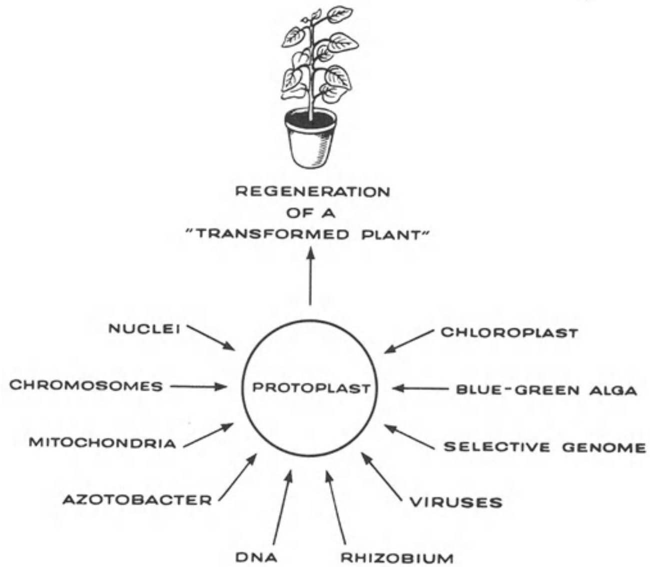

Fig. 6. Hypothetical representation for the uptake of various organelles and introduction of genetic material into protoplasts and the eventual regeneration of a "desired plant"

3.4.5 Chloroplast Implantation

The incorporation of spinach chloroplasts into animal cell cultures was reported by Nass in 1969. These cells divided normally, and the chloroplasts retained their structural integrity. Following this GILES and SARAFIS (1971a, b) implanted *Nitella* chloroplasts into a hen's egg and demonstrated that these chloroplasts survive, remain metabolically active, maintain their morphological integrity, and divide repeatedly. This report on the successful in vitro culture of an organelle reaffirms autonomy of chloroplasts. POTRYKUS (1973) and BONNETT and ERIKSSON (1974) also reported the uptake of chloroplasts by albino *Petunia* and carrot protoplasts respectively, CARLSON (1973a) claimed to have incorporated nonmutant tobacco chloroplasts into the cytoplasm of an albino protoplast. As the albino trait is transmitted cytoplasmically, it is a mutant of the chloroplast-DNA and not of a nuclear gene. These externally supplied "foreign chloroplasts" escaped destruction and entered the cytoplasm; whole tobacco plants were later regenerated from albino protoplasts containing these implanted chloroplasts. Carlson's work has been criticized by POTRYKUS (1973), as white protoplasts from the epidermal cells do give rise to green plants. These protoplasts are white because of their position but green genetically (see also Chap. IV.4). Nevertheless, the transplantation of chloroplasts from one plant to another when extended to plants with a defective or inefficient photosynthetic system could have far-reaching implications in plant breeding.

From the foregoing account it has emerged that (1) an entire plant can be regenerated from an isolated protoplast, and that (2) protoplasts can take up

organelles and genetic materials. Based on this a hypothetical case for the regeneration of a "transformed plant" by a combination of these two steps is presented in Figure 6.

3.5 Induction of Mutations and Genetic Variability

It has been repeatedly observed that plant cells in culture show a wide range of genetic diversity (PARTANEN, 1963; D'AMATO, 1965; TORREY, 1967; DULIEU, 1972; BAYLISS, 1973, see also Chap. III.1). This variability depends on the origin and the genetic constitution of the tissue, and is influenced considerably by physical conditions and growth regulators which induce abnormalities and chromosomal instability. Cells in culture over a long period of subculturing have a tendency to undergo endomitosis, which forms cells with various ploidy levels. These phenomena, though undesirable for maintaining the uniformity and genetic stability of the clones, can be exploited by plant breeders and geneticists for inducing genetic variability in protoplast cultures.

Like callus cells (BAJAJ, 1973), isolated protoplasts (OHYAMA *et al.*, 1974; HOWLAND, 1975; HOWLAND *et al.*, 1975; GALUN and RAVEH, 1975), expecially haploid ones, would make an ideal system (BAJAJ, 1975) for studying the effect of irradiation, and for the induction of mutations by plating them in media supplemented with various chemical mutagens. The main advantage of haploid protoplasts is the high efficiency with which plants with recessive characters can be identified, and mutant lines selected. As with callus cells subjected to mutagens (LESCURE, 1969, 1973; BINDING *et al.*, 1970; WIDHOLM 1972; MALIGA *et al.*, 1973a, b) mutant cell lines from protoplasts of tobacco (CARLSON, 1973b), sycamore (BRIGHT and NORTHCOTE, 1974) and soybean (OHYAMA, 1974) resistant to 5-bromo-deoxyuridine have been obtained.

4. Protoplast Fusion and Somatic Hybridization

One of the most important practical uses of protoplast culture is for somatic hybridization. This could be especially important in sexually incompatible plants, and in cases where conventional methods of breeding fail to operate. Somatic-cell hybridization in animals was achieved by BARSKI *et al.*, in 1960 and later a "man-mouse" hybrid cell was obtained (see EPHRUSSI and WEISS, 1965; HARRIS, 1970). However, this hybrid cell did not form a "Man-Mouse Hybrid". In this connection plant cells have definite advantages over animal systems as they can be grown on fully defined media, and induced to regenerate into an intact plant.

In the following section various methods for the fusion of protoplasts are outlined and an appraisal is made of their merits and demerits for their eventual use in somatic hybridization. Recent progress in the field is also included.

Occasional and uncontrollable fusion of plant protoplasts were reported by KÜSTER as early as 1909, and then by MICHEL in 1939. Following these observations, HOFMEISTER (1954) simply concluded that fusion of plant protoplasts was

an exceptional phenomenon. Since then many workers have employed a variety of techniques to achieve the fusion of plant protoplasts to obtain somatic hybrids.

Protoplast fusion can be classified into two categories: (1) spontaneous fusion, and (2) induced fusion.

4.1 Spontaneous Fusion

Sometimes during the enzyme treatment it is observed that protoplasts from adjoining cells fuse through their plasmodesmata (Fig. 7A) to form a large (Fig. 7B) multinucleate (Fig. 7C) protoplast. Studies with the electron microscope (WITHERS and COCKING, 1972) have shown that enzymatic degradation of the wall removes constrictions on the plasmodesmata permitting their expansion. Such a fusion is called spontaneous fusion, and it is strictly intraspecific. There seems to be a correlation between the size of the leaf and the percentage of protoplasts undergoing spontaneous fusion (USUI *et al.*, 1974); young leaves are more likely to produce such spontaneously induced multinucleate protoplasts.

4.2 Induced Fusion

In contrast to spontaneous fusion, induced fusion of protoplasts does not necessarily involve fusion from the same plant species. In animals, inactivated "sendai" virus is needed to induce cell fusion. However, in plants this type of fusion requires an inducing agent, which first brings the protoplasts together and then causes them to adhere to one another (Fig. 7D). Ultrastructural observations (WITHERS and COCKING, 1972; BURGESS and FLEMING, 1974) have revealed that during fusion there is initially an adhesion and the membranes fuse in small localized areas, then more extensively, and eventually the cytoplasm of the two protoplasts intermingle. Figures 7E–I show both intra- and interspecific fusion of protoplasts of different origin.

4.2.1 Methods of Protoplast Fusion

The isolated plant protoplasts can be induced to fuse with one another by employing several fusogens and procedures, some of which are described here:

Mechanical Method. MICHEL (1939), and SCHENK and HILDEBRANDT (1971) employed mechanical means to bring isolated protoplasts into intimate contact through micromanipulators and perfusion micropipette. This micropipette is partially blocked within one mm of the tip by a sealed glass rod. In this way the protoplasts are retained and compressed by the flow of liquid. By this method, occasional fusions of protoplasts from soybean, *Arachis hypogea* and *Vinca rosea* were observed.

Treatment with Sodium Nitrate. In 1909 KÜSTER observed for the first time that mechanically isolated onion subprotoplasts plasmolyzed with sodium salts underwent fusion on deplasmolysis. Employing the same principle, POWER *et al.* (1970) successfully demonstrated the intra- and interspecific fusion between the

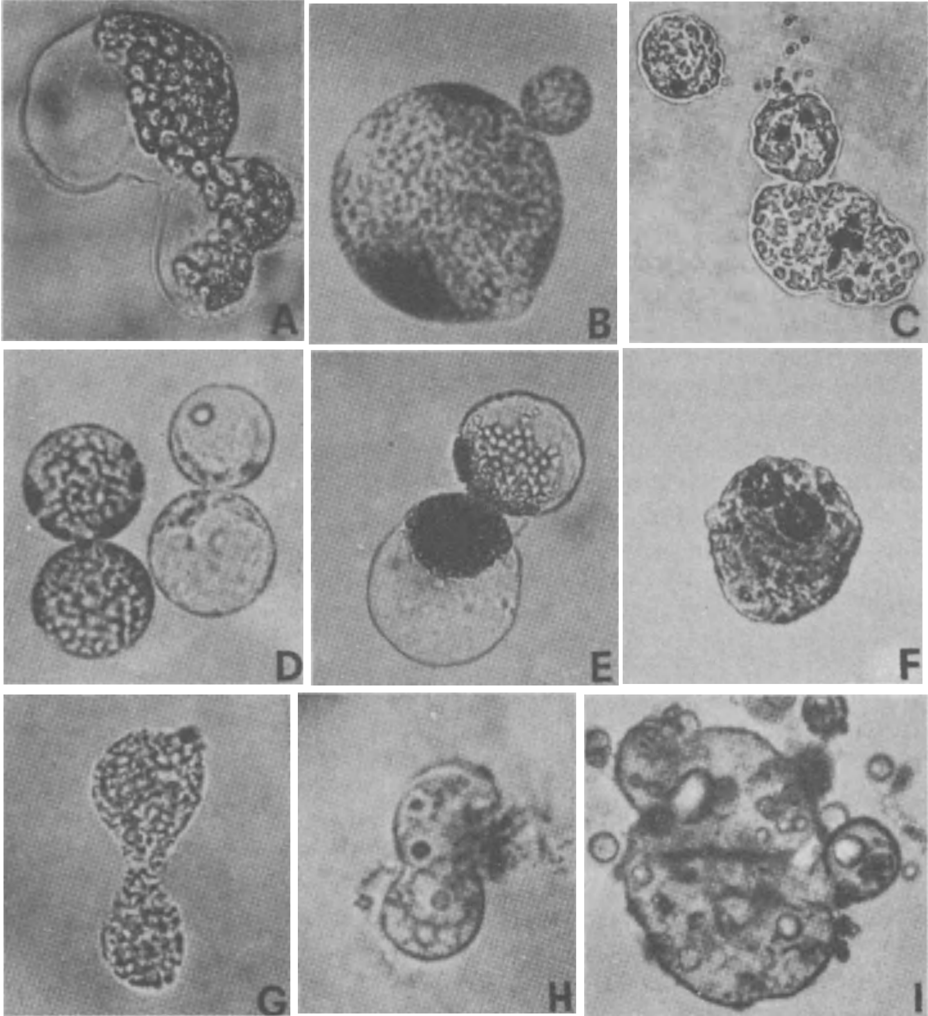

Fig. 7A-I. Fusion of protoplasts. (A) Two plasmolyzed mesophyll cells of haploid *Nicotiana tabacum* cv. White Burley in an enzyme mixture of macerozyme (0.5%) and cellulase (2%) in mannitol (13%). Note the expansion of plasmodesmata and the movement of protoplast from one cell to another, which will eventually form a spontaneous fused protoplast. (B) An extremely large mesophyll protoplast formed as a result of fusion of a number of protoplasts; note the small size of the normal protoplast in the same figure. (C) A uni-, bi-, and multinucleate protoplast of haploid tobacco. (D) A mixture of tobacco mesophyll and wheat cell suspension protoplasts showing adhesion after sodium nitrate treatment. (E) An early stage during fusion event. (F) A binucleate protoplast formed as a result of fusion. (G) Two pollen mother cell protoplasts in the process of fusion after treatment with sodium nitrate. (H) A dump-bell shaped structure obtained by fusion of two pollen protoplasts; note the clearly visible nuclei. (I) A giant protoplast formed as a result of fusion of large number of pollen protoplasts during enzyme incubation. (After BAJAJ and DAVEY, 1974)

protoplasts obtained from root tips of oat and maize seedlings. Their technique involves the following steps: the isolated protoplasts are suspended in an aggregation mixture of 5.5% sodium nitrate in 10% sucrose solution, kept in a water-bath at 35° C for 5 min, and then centrifuged for 5 min at 200 g to obtain a dense pellet. The pellet of protoplasts is once again transferred to a water-bath (30° C) for 30 min. During this time most of the protoplasts undergo fusion. The aggregation mixture is replaced very gently (without disturbing the pellet) by the culture medium supplemented with extra 0.1% $NaNO_3$. The protoplasts are left undisturbed for some time, after which they are washed twice with the culture medium, and plated.

Effect of Proteins on Aggregation. KAMEYA (1973) examined the effect of various proteins on aggregation of protoplasts from *Allium fistulosum*, *Brassica chinensis* and *Daucus carota*, and reported that gelatin and early products of its degradation at a concentration of 2–5% induced aggregation at high frequency within one hour.

Immunological Method. HARTMANN *et al.* (1973) used an immunological method for the agglutination of protoplasts from suspension cultures of *Bromus*, *Glycine*, and *Vicia*. According to this method the protoplasts are tightly agglutinated by immune sera prepared against them in a rabbit. Soybean and bromegrass antibody cross-reacted with and agglutinated *Vicia* protoplasts. These protoplasts were viable, and underwent division after the antibody treatment.

Calcium Ions at High pH. KELLER and MELCHERS (1973) have studied the combined effect of calcium ions at high pH on fusion of tobacco protoplasts. Their method involves spinning the protoplasts in a fusion inducing solution (0.05 M $CaCl_2$ $2H_2O$ in 0.4 M mannitol at pH 10.5) for 3 min at 50 g, and then keeping the tubes in a water bath (37° C) for 40–50 min, when, in most instances, 20–50% of the protoplasts were involved in a fusion event.

Polyethylene Glycol (PEG). The agglutination of protoplasts can be brought about by two methods, depending on the quantity of protoplasts available. (1) When protoplasts are available in sufficient quantities, 1 ml of the protoplasts suspended in a culture medium (WALLIN *et al.*, 1974) is added to 1 ml of 56% solution of polyethylene glycol, and the tube shaken for 5 sec. The protoplasts are then allowed to sediment for about 10 min, washed with the growth medium and observed. Addition of PEG leads to tight agglutination followed by fusion of protoplasts. (2) When microquantities of protoplasts are available drop cultures can be used. Two types of protoplasts are mixed in equal quantities (KAO and MICHAYLUK, 1974; KARTHA *et al.*, 1974a), 4–6 microdrops (100 µl each) are placed in small plastic petri dishes and allowed to settle for 5–10 min at a room temperature. Two or three microdrops, (50 µl) of PEG are then added to the periphery of the mixed protoplast suspension and incubated for 30 min. This causes the protoplasts to adhere to one another and to agglutinate. The agglutinated protoplasts also have a tendency to stick to the surface of the petri dish, but this can be partially avoided by the addition of papain to the medium (CONSTABEL and KAO, 1974). However, we prefer to first place a cover glass in the middle of the petri dish and the microdrops of protoplasts are then poured onto it. This offers

two advantages, (1) it avoids sticking of the protoplasts to the surface of the petri dish, and (2) the protoplasts, while still on the surface of the cover glass, can be fixed, stained and observed.

After PEG treatment, protoplasts are gradually washed, and it is during washing that most of the fusion is accomplished. PEG is then gently replaced by the culture medium. Treatment with PEG causes a high degree of aggregation and fusion, and this frequency is increased by the addition of Ca^{++}.

Protoplasts of different origin show differential sensitivity to PEG (BAJAJ *et al.*, 1975b). For instance a final concentration higher than 25% causes distortion and bursting of the mesophyll protoplasts of *Nicotiana* and *Petunia*, while protoplasts from callus cell suspensions of *Atropa* and carrot remain unaffected. We also observed that agglutination was directly proportional to concentration of PEG, while the viability continued to decrease with increasing amounts of PEG. So, for fusion studies it is necessary to use concentrations of PEG which do not effect viability of the fused products.

PEG has recently been successfully employed to induce fusion between hen erythrocytes and yeast protoplast (AHKONG *et al.*, 1975b) and it seems likely that it might replace viruses for induction of fusion in animal cells.

Miscellaneous. In addition to the above-mentioned techniques, a wide range of additives such as poly-L-ornithine, poly-D-lysine, poly-L-lysine, concanavalin A, cytocholasin B and protamine sulphate (GROUT and COUTTS, 1974), lysozyme (POTRYKUS, 1971b), glycerols and dimethyl sulfoxide (AHKONG *et al.*, 1975a), concanavalin A (GLIMELIUS *et al.*, 1974) have been employed. GROUT and COUTTS (1974) observed that many of the chemicals used by them showed a reduction in electrophoretic mobility of the protoplasts. They considered the enhancement of endocytosis and fusion in terms of electrical repulsion and attraction.

The above-mentioned methods have their own merits and limitations, nevertheless, they have provided a host of information on the mechanism of adhesion and aggregation. They have also paved a way for further intensive studies. One of the most important prerequisites for the successful culture of a "somatic-cell-hybrid" is that the method of fusion and the chemical should not adversely effect its viability, thus rendering it incapable of growth. The use of PEG seems to be most promising as it has the main advantage of being a simple and quicker technique than the serological method, and it causes a high degree of agglutination. Moreover this method does not require the use of high pH, which may be harmful to the protoplasts of plants which are rather more sensitive than tobacco.

4.3 Somatic Hybridization

Both interspecific and intergeneric fusion has been reported by various workers in oat × maize (POWER *et al.*, 1970), *Torenia fournieri* × *Torenia bailloni* (POTRYKUS, 1971a, b), *Brassica chinensis* × *Brassica oleacea* (KAMEYA and TAKAHASHI, 1972) *Vicia*, *Glycine* and *Bromus* (MILLER *et al.*, 1971; HARTMANN *et al.*, 1973), *Lily* (ITO and MAEDA, 1973), carrot (WALLIN *et al.*, 1974), *Atropa belladonna* (BAJAJ, 1974b), *Petunia* (BINDING, 1974b; BAJAJ and DAVEY, 1974) and carrot × tobacco

(GOSCH *et al.*, 1975a). From these studies it can be seen that fusion of protoplasts is a physical phenomenon, and would occur between protoplasts of any two plants. However, it still remains to be seen if there would be an incompatibility reaction among the protoplasts taken from distantly related or unrelated plants. Although this could only be demonstrated by culturing the fused products, the knowledge gained from animal cell fusion indicates, that this is probable, e.g. "man-mouse" hybrid cell line, which continued to grow, even though in some instances the chromosomes of one of the parents were eliminated (HARRIS, 1970).

In animal cell hybridization (MARX, 1973) three results are possible: (1) extinction, in which a phenotype present in one parent cell but not the other is no longer expressed; (2) continued expression of the phenotype in the hybrid, and (3) activation of a phenotype that was expressed in neither parent. WEISS and GREEN (1967) reported that during proliferation of a "man-mouse" somatic cell hybrid there is a complete elimination of human chromosomes. In 1970, KAO and PUCK observed almost the same with a "man-chinese hamster" hybrid cell, where a hybrid cell was available with all the chromosomes of one parent and none from the other. The preferential elimination of the genome can be manipulated by irradiation or BUDR labeling of one of the parent cells before fusion, and this might constitute a useful tool in the use of somatic cells for formal genetic analysis (PONTECORVO, 1971, 1973). If a similar unidirectional elimination of chromosomes occurs in the plant somatic hybrids, or if it can be induced experimentally, it would be an additional means of obtaining genetic variability.

Regarding the culture of plant somatic hybrid cells, GILES (1972) reported the regeneration of a cell wall around a fused protoplast obtained from mesophyll protoplasts of *Digitaria* and those of soybean cell suspension. This demonstrated that not only the isolated protoplasts, but also the fused products can be induced to reconstitute a hybrid cell. Regeneration of a wall, and one to several divisions in the heterokaryocytes between *Brassica napus* × soybean (KARTHA *et al.*, 1974b), barley-soybean, corn-soybean and pea-soybean (KAO *et al.*, 1974) and *Carrot* × *Petunia* (REINERT and GOSCH, 1976) have been reported. CARLSON *et al.* (1972) eventually obtained a "parasexual hybrid" between *Nicotiana glauca* × *Nicotiana langsdorffii*. Briefly, their work involved fusion by sodium nitrate, and when the protoplasts were plated on the medium, only the hybrid cells were able to grow. These workers employed six criteria to establish the identity of the somatic hybrids: (1) morphology of the tissue in culture; (2) morphology of the leaf; (3) trichome characteristics; (4) tumor formation; (5) chromosome number, and (6) patterns of peroxidase isozymes (KUNG *et al.*, 1975). MELCHERS and LABIB (1974) also claimed to have obtained an intraspecific hybrid plant from fused protoplasts of two light and oxygen sensitive mutant strains—Vi-Al (virescent) and Sl_1 Sl_2 (sublethal) of tobacco. Figure 8 schematically represents the sequence of fusion of protoplasts from two different plants, and their subsequent culture and regeneration of "somatic hybrids".

As a means of increasing genetic diversity by somatic hybridization the nuclei from protoplasts of two parents do not necessarily have to fuse, and one could obtain "cybrids" (cytoplasmic hybrids) and also somatic hybrids with some chromosome loss. In this connection callus obtained as a result of protoplast fusion

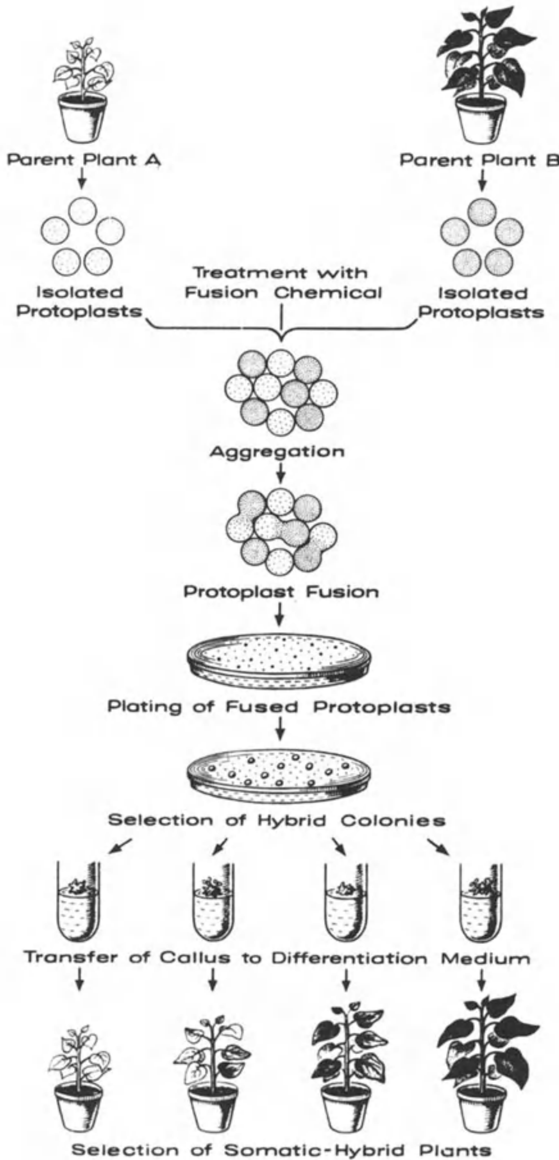

Fig. 8. Diagrammatic illustration for the fusion of protoplasts from two different plant species (here shown with dark and light color leaves), and later plating and selection of hybrid colonies, and the regeneration of "Somatic-Hybrids"

between *Petunia* and *Parthenocissus* (POWER *et al.*, 1975) possessed a peroxidase pattern equivalent to the exact summation of the two parental zymograms, but was not a nuclear hybrid of the two species. Several processes could possibly lead to the formation of such a material which include unidirectional elimination of *Petunia* chromosomes and cytoplasmic hybridization.

If viewed critically, these studies have shortcomings. Nevertheless, they have stimulated research activities in many laboratories around the world, attracted the attention of plant breeders, and have given a powerful impetus to the study of somatic cell hybridization.

GILES (1973, 1974) provided evidence for genetic complementation in a mutant (iojab and white deficient) of *Zea mays* by following changes in the contents of fused protoplasts of redistributed chlorophyll. He observed the greening of the white deficient chloroplast over a period of 72 h. Genetic complementation can be used as one of the markers for the identification of hybrid protoplasts (MELCHERS and LABIB, 1974; GLEBA *et al.*, 1974).

One of the most baffling problems in somatic hybridization studies at present is the availability of a definite marker which could establish the true identity of a "somatic-hybrid". These hybrid cells are difficult to detect because of their rare occurrence. The phenotypic characteristics such as color, size and shape are not sufficient. These characteristics can be inconclusive and even misleading in tissue cultures, since abnormal-looking plants do regenerate from callus cultures. Various biochemical and genetical markers i.e. isozymes pattern, karyotype, chromosome number together with their differential staining, Giemsa and fluorescence banding, drug resistance, pigments, biosynthetic potential, selective nutrient media and genetic variations in cultured cells could be employed. The development of a process for the thorough screening and rigorous selection of hybrid cells needs major priority, as the frequency of successful culture of the fused protoplasts is very low. The recent development of a selection system by COCKING *et al.* (1974) might provide an answer by a complementation-type selection. Their method involves the differential resistance of *Petunia* and tobacco protoplasts to various amino-acid analogues like thioproline and 5-methyl tryptophane (for details see Chap. IV. 2 of this Vol.). The use of these naturally occurring differences in drug sensitivities would eliminate the need for mutant cell lines (COCKING *et al.*, 1974).

The foregoing account, particularly with regard to protoplast culture and somatic hybridization, clearly demonstrates that what was regarded as a fantasy a few years ago is today a reality. Though still in the early stages, this fascinating field of research nevertheless holds a potential of great significance for plant improvement.

5. Conclusions and Prospects

Recent progress in the field of protoplast culture and fusion has established three main points: (1) an isolated protoplast is totipotent and is capable of regenerating a complete plant; (2) protoplasts can be induced to undergo intra- and interspecific fusion to form a "somatic-hybrid", and (3) the pinocytic property of the protoplasts also makes them an excellent material for studies on genetic engineering and cell modification. Although numerous technical problems, still exist, these developments have demonstrated the implications of this discipline of research for plant improvement. Here are some of the aspects which hold promise

for future studies: (1) vegetative propagation by cloning; (2) increase in genetic variability for breeding programs, and the selection of cell lines resistant to various drugs and toxins; (3) induction and easy detection of mutants in haploid protoplast culture; (4) regeneration of "cybrids" and "somatic-hybrids" from fused protoplasts of distantly related, unrelated or sexually incompatible plants; (5) induction of disease-resistance by the incorporation of a selective genome from a disease-resistant into a susceptible protoplast; (6) transplantation of "foreign chloroplasts" into plants with an inefficient photosynthetic system; (7) nuclear transplantation, insertion of a whole or part of a genome, and DNA by uptake and transgenosis; (8) introduction of nitrogen fixing bacteria, blue-green algae, plasmids and "nif-genes" into nonlegumes, especially cereals, might render these plants self-sufficient for increased protein synthesis. These are some of the areas in which the molecular geneticists and agronomists could combine their efforts to improve crop plants.

References see page 563.

2. Selection Systems for Somatic Hybrids

J. B. POWER and E. C. COCKING

1. Introduction

The availability of demonstrable selection procedures for plant somatic hybrids has been restricted to a certain extent by the ease with which protoplasts of species, desired to be crossed somatically, can be induced to divide. In most systems mesophyll tissue is considered to be an ideal source of protoplasts for somatic hybridisation studies, since such protoplasts can be readily isolated. However, it is quite clear that the problem of induction of division in higher plant protoplasts is receding with time.

The basic need for selection arises out of the fact that hybrid cell formation is an infrequent event. Procedures are now available for fusion as a result of which 20–50% of the protoplasts are involved in a fusion event (KELLER and MELCHERS, 1973). Such a fusion event includes protoplast adhesion, but a high proportion of the adhering protoplasts probably do not fuse. The recovery of hybrid material, following selection, and involving an interspecific cross, is exceedingly low. Two considerations are involved; (1) the proportion of heterokaryons that develop into hybrid cells following nuclear fusion, and (2) the survival of these hybrid cells when present at a low frequency within a protoplast population. Plant cells do not survive at very low plating densities, contrary to the situation in many cultured animal cell systems. These factors therefore, all combine to reduce the chances of recovering hybrid material following protoplast fusion, and in the absence of any selection, the probability of recovering hybrid material must be almost zero.

In an apparently ideal system, the intraspecific hybridisation of two varieties of *Nicotiana tabacum* (MELCHERS and LABIB, 1974), where a high fusion frequency was combined with a powerful selection procedure, the recovery of hybrid material was restricted to one event for every 200000 calli developing from protoplasts. This may indeed represent the optimum success rate for the recovery of somatic hybrids and, as more ambitious crosses are attempted, the need for developing powerful selection procedures will further increase.

2. Existing Selection Systems for Plant Somatic Hybrids

All proven selection procedures developed so far have been based upon some knowledge of an existing sexual hybrid and because of this, may be limited in their general applicability. Progress has, in general, been hampered by the lack of suitable biochemical mutants in higher plants. In spite of this, the limited suc-

cesses so far achieved in the field of selection and somatic hybridisation, have served firstly to demonstrate that somatic hybridisation is a reality, and secondly, and perhaps what is more important, that selection procedures must be developed before somatic hybridisation can really advance. The approaches to selection can be broadly classified in two ways.

2.1 Visual Selection

This utilises protoplasts for fusion studies, that are visually distinct at the light microscope level, and it has played an important role in the development of fusion procedures and has allowed limited observations to be made upon the formation and survival of heterokaryon/hybrid cells following protoplast fusion.

Visual selection has, in the main, been restricted to the fusion of colourless protoplasts, isolated from cultured cell or plant epidermal tissues with chloroplast-containing mesophyll protoplasts. Protoplast preparations of different species, bearing the appropriate light microscopic markers, are mixed together, treated with an inducer of fusion such as sodium nitrate, polyethylene glycol (PEG), or Ca^{++}ions buffered at high pH, and following removal of the fusion agent, are examined for heterokaryon formation. Protoplasts which exhibit the fully integrated structural characteristics of both parental protoplast types are thus classified as heterokaryons. During subsequent culture of the fusion products, under non-selective conditions, the development of the heterokaryon, and therefore potential hybrid cells, can be followed. Kao *et al.* (1974) have successfully applied this approach for the fusion of protoplasts of many species. Microscopic observations of fixed heterokaryon material show that nuclei do enter division synchronously but that nuclear fusion does not readily occur, at least during the first few mitotic divisions. Beyond this stage it is technically very difficult to follow further progress, since not only are the cell derivatives of heterokaryons undergoing division but also unfused or homokaryon-derived material of one or both parents. These observations, although curtailed by the lack of adequate biochemical markers in such systems, may provide important clues as to the timing of the application of selection pressure when applied to other systems.

2.2 Selection and the Production of Somatic Hybrids

It was Carlson *et al.* (1972) who first demonstrated the value of a biochemically based selection procedure for the somatic hybridisation of two plant species. The selection theory was based upon a prior knowledge of the nutritional requirements of mesophyll protoplasts isolated from the genetically tumourous amphidiploid hybrid between *Nicotiana glauca* and *Nicotiana langsdorffii.* Protoplasts of the amphidiploid hybrid were able to grow in culture, with a low plating efficiency, to form callus masses, whilst under the same cultutral conditions, protoplasts isolated from both parents failed to develop into calli. This constituted the basis for a selection procedure and, following fusion of protoplasts of the two parent species, somatic hybrid selection would be possible. Protoplasts of the two par-

ents were mixed together and, following treatment with sodium nitrate, were plated in the selection medium. A few calli developed which were subsequently transferred to a culture medium lacking growth regulators, thus further increasing selection pressure against any cells lacking both genomes. Rudimentary shoots, produced on such callus were grafted onto *N. glauca* stock in order to produce whole plants. A comparison made between some of these plants and the sexually derived amphidiploid hybrid, and based on peroxidase isoenzyme pattern, leaf and tissue morphology and chromosome numbers, showed these regenerated plants to be similar to the sexual hybrid and therefore somatic hybrids. It must be stressed that this approach to somatic hybridisation is not generally applicable and cannot be applied to other systems since the hybrid *N. glauca* × *N. langsdorffii* is highly atypical with respect to its nutritional requirements.

Following this demonstration of interspecific somatic hybridisation, MELCHERS and LABIB (1974) have utilised two varieties of *N. tabacum*, bearing chlorophyll defects, in order to produce intraspecific hybrids. These two varieties complement in the sexually derived hybrid to normal green. Haploid, or more accurately "dihaploid" plants (24 chromosomes) of these two varieties, obtained from anther culture, also exhibit chlorophyll defects, and when maintained at a high light intensity, are thus pale in appearance. It was also possible to distinguish the two varieties from one another on a morphological basis since the yellowing response to light was restricted to young leaves only for one of the varieties.

Protoplasts, isolated from leaves, of the light sensitive dihaploid parents were mixed together and fused in the presence of calcium ions buffered at pH 10.5. Selection was based upon the ability of the two genomes, present in the heterokaryon/hybrid, to complement and thus restore normal green colour to differentiating callus and subsequent regenerated plants if maintained at high light intensity. "Several hybrid plants (48 chromosomes) were recovered following this treatment, and upon selfing segregation occurred, since some of the offspring were identical in appearance to the light-sensitive parental types." This also served to confirm that the normal green plants recovered after such a selection were in fact not revertants of either of the parents.

It might be possible to extend this approach of complementation/selection utilising "light sensitive" mutants to other species since chlorophyll defects in plants are not uncommon and are easily detectable. Several basic requirements would, however, have to be met before complementation/selection could be achieved for the somatic hybridisation of other species that are not necessarily sexually compatible. Assuming recessive light sensitive mutants could be obtained for these species, their protoplasts must be capable of division, and ideally whole plant regeneration, when maintained under similar cultural conditions. Although the haploid state is not necessarily a prerequisite for the successful complementation between such mutants no evidence was presented by MELCHERS and LABIB (1974) for the successful somatic hybridisation of diploid light-sensitive mutants of *N. tabacum*. If haploids are essential then this must further extend the basic cultural requirements of species desired to be crossed somatically.

The fusion product of two haploid parents may not be fertile, a problem that may be resolved by a doubling of the somatic hybrid, or alternatively by the use of two diploid parents. The final and most important consideration concerns the

ability with which two light-sensitive mutants of different species could be expected to complement to normal green in the somatic hybrid. Since chlorophyll mutations occur throughout higher plants the chance of the mutation loci and their effects being identical is small, and therefore opportunity for complementation may be great; however it is a requirement for selection that such chlorophyll deficient mutants are light sensitive. However, since the sexual hybrid will not exist, unambiguous proof of complementation capability between such mutants cannot be obtained. Failure therefore to select out somatic hybrids, using light-sensitive mutants, would not necessarily mean that somatic hybridisation was not possible between two such species.

More recently, SCHIEDER (1975b) utilised two strains of *Sphaerocarpos*, bearing auxotrophic recessive non-allelic mutations, to select somatic hybrids. Selection in this system, analogous to selection/complementation in some animal systems, is based upon the restoration of normal nutrient requirements in the somatic hybrid following complementation of the two parental auxotrophic strains.

Protoplasts of the two parental strains, a nicotinic acid deficient, normal green, female, and a glucose deficient, light green, male strain, were mixed together and fused using calcium nitrate buffered at pH 9.0. One plant was recovered, following the culture of fusion-treated protoplasts on a medium lacking both nicotinic acid and glucose. Upon analysis the plant was shown to possess a double chromosome complement in addition to the two heterochromatic sex chromosomes.

Before discussing the possible development of more generally applicable methods for the selection of plant somatic hybrids, it will be particularly useful to consider the methods which have been developed for the selection of animal somatic hybrids. PUCK (1972) has emphasised, with reference to animal cell somatic hybridisation, that there is no other field of science that promises as much excitement and accomplishment in the next decades. What particular attributes are possessed by cultured animal cells which have made selection so much easier and, can these selection procedures be adapted for use with plant cells?

3. Existing Systems for Animal Somatic Hybrids

As emphasised by PONTECORVO (1974) hybrids between birds and mammals have never been produced and only mammalian somatic hybrids are probably obtainable. Even with mammalian cells and combined with the most favourable conditions of fusion, using Sendai virus, the frequency of formation of somatic hybrid cells that one can obtain is of the order 10^{-3} to 10^{-4}. Therefore it is necessary to use some system of selection for hybrids, since they could not be detected among the cell population.

The first selective system for the isolation of mammalian somatic hybrids was based on the demonstration by LITTLEFIELD in 1964 that one can, as in microbial genetics, establish a selective system wherein the hybrid cells will grow and the parental cells will not. As pointed out by EPHRUSSI (1972), this experiment, which utilised two sublines of a heteroploid mouse line, provided the first demonstration

of the occurrence of complementation in hybrids of mammalian cells with the continued production in the hybrid cells of two enzymes, each of which is synthesised by only one of the parents and involved dominance at phenotypic level of the normal condition over the enzymatic deficiency.

Mammalian somatic hybrid cell lines can only be obtained readily if parental cells are utilised which possess discernable cell markers and which are capable of being selected against. Fusion of the parental cells containing different selection markers is then induced, and after a short period of culture in the complete medium the selective medium is applied. Under this selection pressure each parental cell line should be eliminated and the hybrid cells should grow and divide since the parental defects are complemented in the hybrids. As fully discussed by GILES and RUDDLE (1973) such systems used to select hybrid cell lines from mixed populations of fused cells and parental lines may be separated into two general categories (1) biochemical methods and, (2) methods based on growth characteristics and morphology. Combinations of these two are also frequently employed. Biochemical methods involve, in the main, the use of drug resistant mutants, nutritional auxotrophs and temperature-sensitive mutants.

3.1 Drug Sensitivity

Selection using drug-resistant mutants has principally involved the use of cell lines which are resistant to the antagonists of purine and pyrimidine synthesis, 8-azaguanine (8-AZG) and 5-bromodeoxyuridine (BUDR), and therefore are susceptible to the action of aminopterin. This is the basis of the LITTLEFIELD selection procedure, and consequently if one hybridizes a cell which is 8-AZG-resistant and aminopterin-sensitive with one which is BUDR-resistant and aminopterin-sensitive, and grows the resulting mixed population on a medium containing hypoxanthine, aminopterin and thymidine (HAT), both parental cell lines should die but the hybrid cells should grow due to complementation of the deficiencies in the hybrid (for a detailed discussion see GILES and RUDDLE, 1973). Half-selective systems can also be employed. DAVIDSON and EPHRUSSI (1965) have shown that by using the LITTLEFIELD selection medium it is possible to obtain hybrids between heteroploid cells carrying a selective marker (i.e. drug resistance to 8-AZG in L cells) and normal diploid cells by making use of the fact that normal cells grow relatively slowly.

Diploid strains of Chinese hamster cells, selected for resistance to aminopterin and actinomycin D have also been employed in cell hybridisation studies (SOBEL *et al.*, 1971). Hybrids were selected by their ability to grow in the presence of both aminopterin and actinomycin D whereas the parental lines could not. In a similar way resistance to the cardioactive steroid ouabain, which affects the membrane sodium-potassium activated ATPase has also been employed in hybridisation studies. It is readily possible to isolate mutants resistant to ouabain from hamster or L cells, and the mutants are stable (BAKER *et al.*, 1974). As noted by GILES and RUDDLE (1973) it has been shown that human cells are killed at very low concentrations of ouabain (10^{-7}M) while the lethal dose for mouse cells is substantially higher (10^{-3}M). Consequently for the isolation of hybrid cells from mouse ×

human cell fusions it may not be necessary to search for ouabain resistant mutants in mouse cells.

Cell hybridisation has been used to test the dominance or recessiveness of the ouabain resistance mutation and it has been concluded that ouabain resistance is a dominant or co-dominant mutation (BAKER *et al.*, 1974). Ouabain-resistant Chinese hamster cells were selected and fused with ouabain-sensitive Chinese hamster cells and the hybrids were found to be much more resistant to ouabain than were the sensitive parental cells. Somewhat similarly actinomycin D-resistance in hamster cells has been shown to be dominant or co-dominant in somatic hybrids. However, as pointed out by DAVIDSON (1974) it should not be assumed that membrane-associated resistance to such extrinsic agents is always expressed in hybrids between sensitive and resistant cells. For instance hybrids between human and mouse cells are sensitive to polio virus whereas the mouse cells are naturally resistant.

3.2 Auxotrophic Mutants

Auxotrophic mutants have also been utilised for selection in somatic hybridisation and their use has enabled complementation analysis to be readily carried out between cells that differ from each other in single-gene mutations only. These fusion and selection experiments have made it possible to decide whether such mutations are recessive or dominant. PUCK (1972) has described the fusion of a glycine-requiring (gly^-) and hypoxanthine-requiring (hyp^-) mutant of a Chinese hamster ovary culture. Following fusion (approx. 2% of the cells) aliquots of the cells were plated in media lacking the two critical nutrients. It was found that whereas each mutant alone exhibited no growth unless its specific nutritional requirement was supplied, about 1% of the cells plated after fusion in the medium lacking both nutrients grew to colonies which bred true. Thus mutations to glycine and hypoxanthine requirements are recessive since the fused cells show neither deficiency. In general it would appear, from hybridisation experiments, that all nutritional mutants of cultured animal cells are recessive.

3.3 Temperature-sensitive Mutants

Most temperature-sensitive (*ts*) mutations are recessive in viruses, bacteria, fungi and *Drosophila*. In the case of *ts* mutants of cultured mammalian somatic cells we are of course dealing with diploid organisms and it might perhaps be thought likely that such mutations would be dominant. However, somatic hybridisation between *ts* mutants with resultant complementation has, as for auxotrophic mutants, clearly shown that most or all of the mammalian cell *ts* mutants are recessive (BASILICO and MEISS, 1974). Although the temperature optima of various cells which grow in tissue culture can occasionally differ greatly, this naturally occurring difference has not as yet been exploited for selection (STEPLEWSKI and KOPROWSKI, 1970).

Thus we see that biochemical methods can be employed for the selection of somatic hybrids by complementation of auxotrophic or *ts* recessive mutants.

A central question, which has been highlighted by PUCK (1972), now arises and this concerns the genetic nature of the requirement for a growth factor or a biochemical lesion associated with an enzyme defectiveness in a presumably diploid cell. As pointed out by PUCK "the probability of two independent mutations at each of the two sister genes in homologous chromosomes is too small to be seriously considered". It is of course true that a single dominant mutation could produce an auxotrophic nutritional requirement or a *ts* mutation, but this probability has been ruled out by complementation studies. Sometimes it would appear likely that some chromosomal material is missing and as a result the genes in this missing region are present in the hemizygous state. Consequently a mutation in any of the genes of this hemizygous region would produce a phenotypic change even if the mutation were recessive. As THOMPSON and BAKER (1973) have emphasised "in view of the increasing variety of lines of altered phenotype which are now being isolated it is no longer clear that the problem of ploidy is a major obstacle to obtaining mutations affecting the whole range of cellular functions".

Observations on acquired drug resistance, many of which are dominant or codominant, are also difficult to reconcile with the usual explanations based on gene mutation and chromosomal change. HARRIS (1974) has suggested that the underlying mechanisms involve alteration in gene expression rather than changes in genetic information.

Other methods for the selection of somatic hybrids of mammalian cells have in the main used growth characteristics when the elimination of both parents using biochemical methods was either not possible or undesirable (AYAD and DELINASSIOS, 1974). These methods have involved aspects of cell phenotype such as growth rate, contact inhibition, adhesion to glass or plastic and general cell morphology. As indicated earlier such growth characteristic differences have been used in combination with biochemical methods in half-selective methods.

4. The Development of Generally Applicable Methods for the Selection of Plant Somatic Hybrids: Problems and Perspectives

Can we apply the generally applicable selection procedures which work for the selection of mammalian somatic hybrids to the selection of higher plant somatic hybrids?

MALIGA *et al.* (1973b) and LESCURE (1973) have reported on the growth of tobacco callus cells in BUDR agar medium, and BRIGHT and NORTHCOTE (1974) have succeeded in isolating a mutant tissue of sycamore selected for by its resistance to BUDR. OHYAMA (1974) has utilised protoplasts, isolated from cultured soybean cells to demonstrate comparable resistance. These workers have been particularly interested to determine whether such BUDR-resistant cell lines could be utilised in a HAT type selection procedure. Unfortunately both the soybean BUDR-resistant cells and the sycamore BUDR-resistant cells appear to have both thymidine uptake systems and thymidine kinase activity. Even though these

BUDR-resistant cells were found to be sensitive to the HAT medium, enzyme deficiency complementation, comperable to the LITTLEFIELD HAT system, using such cell lines, would probably therefore be unlikely. LESCURE (1973) isolated an azaguanine-resistant tobacco callus which might also be useful in the HAT system but unfortunately the basis of the resistance was not established. More recently BRIGHT and NORTHCOTE (1975) have isolated a mutant callus of sycamore which had a 10-fold increase in resistance to azaguanine when compared with the wild type. This increased drug resistance was shown to be accompanied by a 50% reduction in the normal level of hypoxanthine phosphoribosyltransferase (HGPRT). Unfortunately this reduction in HGPRT activity is not sufficient to enable such a cell line to be utilised in the HAT system.

WIDHOLM (1974) has highlighted the unlikelihood of isolating auxotrophs from diploid plant cells and it would seem unlikely that recessive auxotrophic mutants of diploid higher plant cells will become available for complementation selection following the fusion of higher plant protoplasts. Somewhat comparable conclusions can probably be reached regarding the isolation of temperature sensitive mutants and their use in complementation selection. It is clear that haploid protoplasts or cells will probably need to be used for such studies of the isolation and utilization of auxotrophic and *ts* mutants.

Morphological markers are rare in cultured plant cell systems. Such a marker is the differential greening of colonies which could offer an opportunity for selection.

The isolation of suitable mutant cells of higher plants is further beset with the difficulty of culturing plant cells at low cell density. The minimum cell density is usually about 10^3 cells/ml. Selected cells, although present initially, may be unable to grow adequately and therefore may not be detected unless a long-term series of selective enrichments is employed. When selecting for drug resistance, if the resistance is dominant, then the chances of isolating such mutants from cultures of diploid higher plant cells are much greater. However, once again the inability of these cell cultures to grow at very low cell densities may result in negative results. Because of these difficulties when attempting to work with mutant cell lines attention here in the ARC Group at the University of Nottingham has centred on the possibility of utilising naturally occurring differences in the drug resistance of different species (COCKING *et al.*, 1974). For instance, without recourse to the isolation of mutant cell lines, it is possible to detect approximately 10-fold difference in the resistance of two higher plant species (in this instance *Petunia* and *Nicotiana*, see Sect. 4.1) to the amino acid analogue azatryptophan and to abscisic acid. This is comparable to the 10-fold increase in resistance to azaguanine reported by BRIGHT and NORTHCOTE (1975) for their mutant sycamore callus. This detailed survey of over twenty different drugs was prompted by the report of somewhat comparable differences in the resistance of cultured mammalian cells to ouabain. As we have seen earlier with respect to ouabain, the drug resistance was shown to be dominant following fusion with a sensitive species. If comparable dominance can be demonstrated with the higher plant protoplast system then complementation selection in the presence of two drugs could be used to assess the feasability of such selective systems for the isolation of somatic hybrids.

4.1 Selection Using Non-mutant Cell Lines

Many of the practical problems, previously envisaged when considering the somatic hybridisation of plants, have been resolved, but only for certain systems. The somatic hybridisation of two closely related, yet sexually incompatible, species such as *Petunia* and *Nicotiana* is considered by many to be the next major development in the field of somatic hybridisation. There is also every reason to assume that the approach leading to the successful somatic hybridisation of two such species could be generally applicable.

In the absence of the sexual hybrid, certain assumptions have to be made concerning the selection processes that may lead to the production of a somatic hybrid between species of the two genera. Protoplasts, in general, will grow and divide under comparable cultural conditions, and for some species within the genera, result in the regeneration of whole plants. It is therefore assumed that the somatic hybrid will also survive under conditions supporting the growth of both parents. The major hurdle is the development of selection procedures that are based upon firmly established biological principles.

In the absence of suitable mutant cell lines, the obvious approach is to develop a selection procedure based upon naturally occurring differences between species of these two genera. In this respect it has been shown that certain species of the two genera differ markedly in their resistance, at the protoplast level, to drugs such as amino acid analogues and antiauxins (COCKING *et al.*, 1974). Once these differences have been established it is equally important to demonstrate that drug resistance is dominant for the species concerned. In the absence of the intergeneric sexual hybrid, dominant resistance can be determined for a given species, with respect to an interspecific hybrid within the genus. In animal systems, dominant drug resistance can be successfully applied in complementation/selection for somatic hybrids and moreover, dominant resistance to a particular drug appears to be consistent even for different species.

This concept, applied to plant somatic hybrid selection would alleviate the search for mutant cell lines. Somatic hybridisation could therefore be achieved using protoplasts isolated directly from the normal plant, which can only mean that the realisation of the full potential of somatic hybridisation may come quite soon.

In conclusion therefore, it can be seen that many problems exist with respect to plant somatic hybridisation and the development of selection systems. Cultured animal cells with their inherently low level of biological competence are ideal systems for the production and isolation of auxotrophic, temperature-sensitive or drug-resistant mutants, which in turn are suitable for complementation/selection studies. Unfortunately, this is not the case when considering cultured plant systems. However, many parallels in the development of selection systems, including the HAT system and the production of auxotrophic mutant cells using haploids, may provide an inroad into the successful establishment of selection systems for plant somatic hybrids.

References see page 563.

3. Cell Modification by DNA Uptake

D. HESS

1. Introduction

As far back in history as can be traced, man has tried to improve on nature or has at least attempted to bring about alterations that in his opinion justify the attempts. This holds true for the manipulation of the genetic material of animals as well as of plants. The aim of genetic manipulation is the combination of genic material from different sources; in higher plants the natural mechanisms of pollination and fertilization impose strict limitations on this objective. Some of these could be overcome, e.g. incompatibility barriers by in vitro fertilization. For instance, in the genus *Brassica* (KAMEYA *et al.*, 1966), in the Caryophyllaceae (ZENKTELER, 1967) and—with a somewhat modified technique—in the Gramineae (CARLBOM, 1969), interspecific combinations could be obtained by sprinkling the pollen directly onto the ovules. But in these cases, only close relatives could be combined. New techniques are now being tested to combine the genetic material of systematically unrelated species, techniques sometimes so sophisticated that the expression "genetic engineering" seems to be justified (LEDOUX, 1971, 1975; RASPE, 1971; HESS, 1972a, 1974, 1975a, b, c; COCKING, 1972; CHALEFF and CARLSON, 1974; HEYN *et al.*, 1974; JOHNSON and GRIERSON, 1974; MERRIL and STANBRO, 1974; SMITH, 1974; KLINGMÜLLER, 1975). A common feature of all these new methods is that a DNA receptor has to be combined with a DNA donor (in this article the term DNA donor is used not for the whole organism delivering the genetic material to be transferred, but for the DNA carrier combined with the DNA receptor). For reasons of survival the DNA receptor must possess a certain level of organization, while in the case of the DNA donor one could even use "pure" DNA.

Table 1 lists the various receptor-donor systems used so far. First of all, in these systems the uptake of exogenous genetic material must be proven. Secondly, expression of the introduced foreign genetic material must be demonstrated. Expression would involve transcription and translation and could be tested on these levels, but also by the appearance of more complicated new "biological" characteristics. Thirdly, there should be integration of the exogenous genetic material into the genome or plastome of the DNA receptor. Data on integration are very scarce. The same holds true for the replication of the exogenous genetic material—a prerequisite that must be fulfilled if the new genetic information is to be maintained through several generations.

Let us consider various DNA receptor-donor systems and their suitability with regard to the uptake, expression, and—if possible—integration and replication of foreign genetic material. Some systems, dealt with in Chapter IV of this volume (e.g. protoplasts + cell organelles, protoplasts + bacteria, and protoplasts

Table 1. DNA donor–receptor systems used in genetic manipulations with higher plants

DNA receptor	DNA donor	Uptake	Expres-sion	Inte-gration	Repli-cation	Trans-location
Cell organelles (Nuclei)	bacterial DNA	p. 508	p. 508	—	—	—
Protoplasts	plant DNA	p. 509	—	—	—	—
	bacterial DNA	p. 509	—	—	—	—
	plasmids	—	p. 512	—	—	—
	phages	p. 511	p. 512	—	—	—
	cell organelles, cf. Chap. IV					
	protoplasts (somatic hybridization), cf. Chap. IV					
Cells and tissues in culture	plant DNA	p. 512	p. 513	—	—	—
	bacterial DNA	p. 512	—	—	—	—
	phages	—	p. 514	—	—	—
Cells in organisms (embryos, seedlings, plants)	plant DNA	—	p. 520	p. 524	—	—
	bacterial DNA	p. 517	p. 523	p. 524	p. 528	p. 518
	phages	p. 517	p. 517	—	p. 517	—
Egg cell or early Embryo (pollen as carrier)	plant DNA	—	p. 530	—	—	—
	bacterial DNA	p. 529	p. 531	—	—	—
	plasmids	—	p. 531	—	—	—
	phages	p. 530	p. 531	—	—	—

+ protoplasts) are listed in Table 1 without any further discussion. Virus/plant relationships are treated in Chapter V.1.

In connection with the present survey the question arises as to whether in vitro techniques need be used at all. If one considers the well-known difficulties for the regeneration of plantlets by cells of higher plants growing in vitro, it appears simple to incorporate the exogenous genetic material into intact organisms. These should be embryos or plantlets that after the incorporation of genetic materials could grow into plants with complete or partial genetic changes. This technique, however, cannot be used for the bacterial genes because the presence of bacteria has to be excluded which is best accomplished by aseptic culture. In principle any higher plant can be raised in the test tube from seed to mature plant under aseptic conditions. However, there are practical reasons (e.g. the size of the culture vessels) which make it impossible. A few exceptions to this rule exist, for instance the tiny crucifera *Arabidopsis*. With plants of normal size only the early stages of development could be kept under sterile conditions and the plants have to be transferred later into the soil. Therefore, the necessary testing for the appearance of bacterial genes in the higher plant has to be done during the relatively short period of in vitro cultivation.

If work is done in vitro it is possible to use pieces of tissue or cells in suspension culture for treatment with DNA instead of seeds and plantlets. However, this would not fully explain the present tendency to use in vitro techniques since there is no fundamental difference between treating cells in an embryo, and in a cell cluster of a suspension culture with DNA. Possible advantages gained by the use

of cell cultures may even be lost by the difficulties encountered in some species when raising large numbers of progeny. In transformation experiments with DNA, since there is a very low probability that genes are transferred into a cell, a very high number of progeny is needed. A point clearly in favor of the use of in vitro techniques is the use of isolated protoplasts: the removal of the wall renders the cell more capable of accepting foreign materials. Whether or not this hypothesis is valid is an open question. Another point worth mention is that more plants can be raised from protoplasts of different species (see Chap. IV. 1), such experiments however have been unsuccessful with many species. In addition, the rate of regeneration is frequently so low that even in the case of an enormously increased DNA uptake it would be pure chance if one of the regenerated plants were genetically changed. Perhaps the system described at the end of this article, in which pollen treated in vitro with exogenous DNA and subsequently carried during the natural pollination and fertilization, offers some advantages. Further, in the case of seeds, high numbers of progeny could be obtained. Seeds could be germinated in vitro and if contaminated could be tested for bacterial genes under sterile conditions. However, as of this writing, this technique has not been tested. The situation would be even better if the DNA were incorporated into the early developmental stages, i.e. microspores; in these experiments one could expect to raise haploid plants with complete genetic changes.

In this article it is attempted to discuss the reasons for the present trends to use in vitro techniques. However, the data collected from in vivo and in vitro experiments are so interdependent that they have to be evaluated together.

2. Isolated Cell Organelles as Receptors

In genetic manipulations one intends to create whole plants with new characteristics. Under this aspect, of course, isolated cell organelles are of no value as receptors of exogenous genetic material, but they offer the possibility of testing the utilization of the foreign gene material in an in vitro system. There is no doubt, that one could also work with an organelle-free in vitro system. RICHTER *et al.* (1972), for example, demonstrated that a system isolated from yeast mitochondria was able to transcribe T 3 phage DNA and to translate the messenger formed into the phage-specific enzymes lysozyme and S-adenosylmethionine cleaving enzyme. Nevertheless, whole organelles are easier to handle and allow more conclusions to be drawn regarding the behavior of the whole cell, the nucleus being perhaps the most interesting.

Nuclei of *Petunia* and *Nicotiana* were isolated by triton X-100 from protoplasts. They showed a 10–100 fold higher transcription activity than nuclei prepared by conventional methods (BLASCHEK *et al.*, 1974). This high transcription activity was the prerequisite for testing the utilization of exogenous DNA. Isolated nuclei of petunia were incubated with *Escherichia coli* DNA. As revealed by DNA/RNA hybridizations the bacterial DNA was transcribed. Transcription takes place either on the surface of the nuclei or in the surrounding medium.

Rifamycin does not impair the transcription of the bacterial DNA indicating that plant polymerases must be at work. Part of the exogenous DNA may penetrate into the nuclei, but this is not yet conclusively proven (HESS, 1976a; BLASCHEK and HESS, 1976).

The system is easy to practice and shows that foreign DNA can be used by the plant system. This is important because it has not been proven that the plant enzymes, e.g. the polymerases involved in transcription and replication, could work with any foreign DNA.

3. Protoplasts as Receptors

3.1 Uptake

In this and in the following sections only uptake experiments in sensu strictu are treated. Experiments where one might conclude from an expression that foreign genetic material was previously taken up will be dealt with in the sections on expression. Isolated protoplasts, wall-free cells, seem to be best suited for the introduction of foreign genetic material. Isolated protoplasts of several species, *Nicotiana tabacum* (NAGATA and TAKEBE, 1971), *Daucus carota* (GRAMBOW *et al.*, 1972; KAMEYA and UCHIMIYA, 1972), *Petunia hybrida* (HESS *et al.*, 1973), *Asparagus officinalis* (BUI-DANG-HA and MACKENZIE, 1973), *Sphaerocarpos donellii* (WENZEL and SCHIEDER, 1973), *Datura innoxia* (SCHIEDER, 1975a) and *Atropa belladonna* (GOSCH *et al.*, 1975b) have been regenerated to whole plants (for more details see Chap. IV.1). Therefore, one essential requirement for the use of protoplasts in genetic manipulation experiments is available. Protoplasts apparently engulf what is brought in contact with them: TMV (TAKEBE and OTSUKI, 1969), TMV-RNA (AOKI and TAKEBE, 1969), proteins (HESS, 1973a) polystyrene latex particles (MAYO and COCKING, 1972), perhaps plastids (BONNETT and ERIKSSON, 1974) and nuclei (POTRYKUS and HOFFMANN, 1973), and even other protoplasts (HESS, 1975a). No doubt, DNA, plasmids and phages could also be incorporated. Nevertheless, it is rather difficult to prove this uptake especially with DNA.

3.1.1 Uptake of DNA

The first uptake studies were inconclusive. Protoplasts isolated from several species were fed with ^{4}H-labeled *E. coli* DNA, and radioactivity was found in TCA precipitates obtained from these protoplasts (OHYAMA *et al.*, 1972). Autoradiographic studies with petunia protoplasts fed with ^{14}C-labeled *Petunia* DNA demonstrated radioactivity, especially in the area of the nuclei (HOFFMANN and HESS, 1973). In both cases, however, complete degradation of the foreign DNA during or after uptake could not be excluded.

In further studies on the uptake of exogenous DNA, petunia protoplasts were incubated under sterile conditions with ^{3}H-labeled DNA isolated from a thymine-less strain of *E. coli* (HESS, 1976a; LIEBKE and HESS, 1976). After incubation

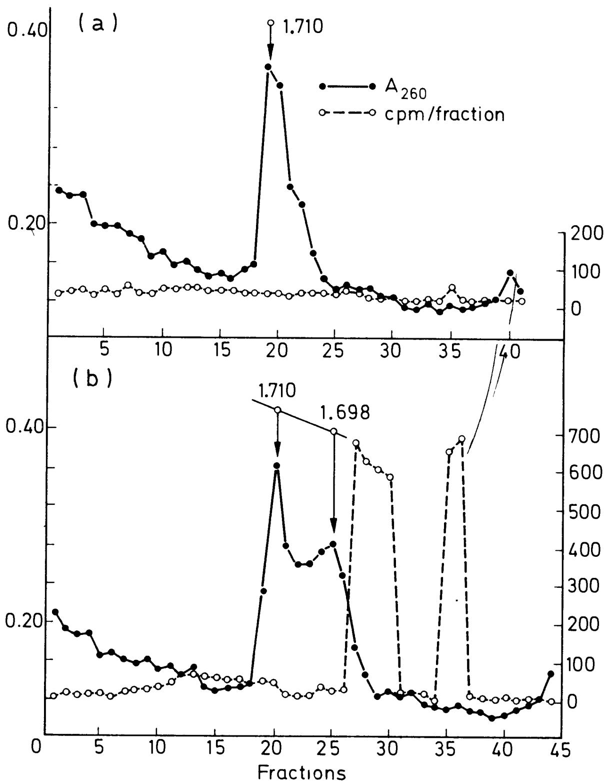

Fig. 1a and b. Effect of treatment with DNAase I on the DNA extractable from isolated protoplast nuclei. Incubation of mesophyll protoplasts of *Petunia hybrida* with 3H-labeled *E. coli* DNA (20 μg/ml, spec. activity 30000 dpm/μg), isolation of the protoplast nuclei, CsCl density gradient centrifugation of the DNA extracted from these nuclei. Density marker: *E. coli* DNA (50 μg/gradient). *E. coli* DNA $d = 1.710$ g/ml, *Petunia* DNA $d = 1.698$ g/ml. (a) DNA from 8.5×10^6 nuclei treated with DNAase I, (b) DNA from 4.5×10^6 nuclei not treated with DNAase. The nuclei were isolated from the same protoplast preparation following the DNA incubation (HESS, 1976c)

the protoplasts were washed and treated with DNAase I. So contaminations by the DNA in the medium could be excluded, but not those by DNA fixed on the protoplast surface in the form of DNAase-insensitive complexes. To eliminate this possibility, the nuclei were isolated by a mild treatment with triton X-100, washed and DNA was extracted from the nuclear fraction. DNAase treatment of the nuclei has to be ommitted (cf. below). In the CsCl density gradient, labeled DNA could be detected, sometimes with approximately the buoyant density of the DNA fed, and sometimes with a lower one (Fig. 1). As demonstrated in experiments where the protoplasts were incubated with ^{3}H-labeled thymidine, there was no DNA synthesis during the incubation period. So the conclusion seems to be justified that we were dealing with the bacterial DNA fed that might undergo minor or major modifications, as indicated by the changes in its buoyant density. One further objection had to be taken into consideration i.e. triton does not

destroy DNA under the conditions used. So the bacterial DNA detected could be derived from DNAase-insensitive complexes on the protoplast surface. Triton might have disintegrated these complexes and the DNA released might have been adsorbed by the nuclei during the isolation procedure. But the yield of reextractable DNA from our nuclear fraction could be greatly enlarged by poly-L-lysin. Polykations stimulate the uptake of exogenous material, e.g. TMV-RNA (AOKI and TAKEBE, 1969), proteins (HESS, 1973a) into protoplasts. Furthermore, the yield could be highly improved by feeding the DNA as Ca phosphate suspension, a method described for the enhanced uptake of *Adenovirus* 5 DNA (GRAHAM and VAN DER EB, 1973). The efficacy of well-known uptake stimulants leaves no doubt that we are dealing with an uptake phenomenon. However, the experimental procedures used make it difficult to determine whether this uptake took place only into the cytoplasm or into the nuclei as well.

In previous studies HOFFMANN (1973), using the same method, claimed that after feeding petunia protoplasts with double-labeled *Petunia* DNA, the nuclei had taken up the exogenous DNA. Unfortunately, he overlooked the fact that triton does not destroy DNA, so that the DNA he found might have been derived from the above-mentioned DNAase-insensitive complexes. Furthermore, he treated the nuclei isolated from the protoplasts with DNAase I. In our experience, DNAase I penetrates rapidly into the nuclei under the conditions used by HOFFMANN (1973) and destroys not only the foreign DNA but also the nuclear DNA. One might wonder then whether HOFFMANN could detect any DNA at all. Another disadvantage was the use of CsCl-ethidum bromide density gradients in such a way that no conclusions as to the maintenance or modification of the exogenous DNA could be drawn.

The DNA uptake studies in the protoplast system were more extensively discussed because they demonstrated the difficulties encountered even in a comparatively simple system. Undoubtedly, these difficulties must increase in more complex systems such as cells in culture or organisms. Scepticism seems to be justified in such cases where an uptake without any difficulties was claimed.

3.1.2 Uptake of Phages

The success of MERRIL *et al.* (1971, 1972; MERRIL and STANBRO, 1974) in their phage-fibroblast system stimulated comparable work in the phage-protoplast system. As far as uptake studies are concerned only data for petunia protoplasts are available (HESS, 1976a; JENNE and HESS, 1976). *Petunia* protoplasts were incubated with λ-phages and then washed till there was no further decrease in the phages was detected in the washing fluids by the plaque test. The washed protoplasts were extracted. They produced more pfu (ca. 100 per protoplast) than were present in the last washing fluid. Poly-L-lysin stimulates the yield of pfu isolated from the protoplasts so that once more we are dealing with uptake processes and not with a mere superficial adsorption. Investigations as to whether the phage material could be taken up into the nuclei are in progress.

3.2 Expression

Data on the expression of exogenous genetical material in isolated protoplasts, or in cells derived from these protoplasts, have been rather rare and not always

sufficiently substantiated. For instance, in several preliminary reports it turned out that proper controls were omitted.

A more biochemical approach was taken by CARLSON (1973a). In protoplasts of *Hordeum vulgare* infected with the coliphage T3, synthesis of two phage specific enzymes (a S-adenosylmethionine cleaving enzyme and a RNA polymerase) normally not produced by *Hordeum* could be demonstrated. Unfortunately, experimental details are lacking.

Other approaches of more biological nature were based on growth tests. Essential supposition of such a growth test must be that the protoplasts that take up the exogenous genetic material are still able to divide. Cell division and callus formation in protoplast cultures treated with DNA and phages were obtained in petunia (HESS, unpublished results). Usually the percentage of dividing protoplasts is comparatively low, and even if it could be as high as 80–90% (BINDING, 1974a) one could argue that the protoplasts that had taken up the exogenous genetic material might be among the nondividing 10–20%. Therefore, attempts were made to introduce R-factors against kanamycin into a cell wall-free mutant of *Chlamydomonas reinhardii*, a model protoplast with rapid and 100% division (GRESSHOFF and HESS, 1976). Recently, LURQUIN (1975) demonstrated an uptake of exogenous DNA in *Chlamydomonas*. Following incubation with R-factors isolated from a kanamycin-resistant strain of *E. coli*, several kanamycin-resistant clones of the alga could be selected. Treatments with control DNA and with buffer remained without any effect. Unfortunately, the resistance was lost after 40 *Chlamydomonas* generations. This may be due to difficulties in the integration of the plasmid.

4. Cells and Tissues in Culture as Receptors

4.1 Uptake

Uptake of exogenous DNA into tobacco cells in shake cultures was studied by BENDICH and FILNER (1971). The tobacco cells were separately incubated, with ^{32}P-labeled *Pseudomonas aeruginosa* DNA and with "heavy" ^{3}H-labeled tobacco DNA. From the cells, broken up by mechanical means, a pellet was obtained which was rich in nuclei and nuclear fragments. From this fraction DNA was isolated and tested by CsCl density gradient centrifugation. 0.5% of the exogenous DNA initially fed could be recovered in a high molecular weight, albeit partially degraded state, provided that DNAase secreted from the cells into the medium was first removed by treatment with pronase. Unfortunately, the cells were not treated with DNAase to remove externally adsorbed DNA, and no precautions were taken to prevent contamination of the nuclear fraction with this DNA or with DNA derived from complexes with surface constituents (cf. Sect. 2.1). So one has to consider the results with some reservation. In comparison, the advantages of the protoplast system just discussed are quite obvious.

Let us now include the work with fungi. OJHA and TURIAN (1971) studied the uptake of ^{32}P-labeled *Allomyces* DNA into meiospores of *Allomyces*. Before DNA

extraction the meiospores were carefully washed and treated with DNAase. DNA was extracted following the method of BRITTEN *et al.* (1969). From the results obtained it was concluded that a minor amount of the DNA fed was incorporated into the recipient genome.

4.2 Expression

Data on the expression of exogenous DNA in cultured cells are more convincing than those on the uptake. For instance, in the above-mentioned *Allomyces* system the position of the gametangia could be changed. Normally, *Allomyces macrogynus* is epigynous; however following treatment of its meiospores with DNA of the hypogynous *Allomyces arbusculus*, some hyphae showed the hypogynous character (Fig. 2). The reverse experiment (meiospores of *A. arbusculus* treated

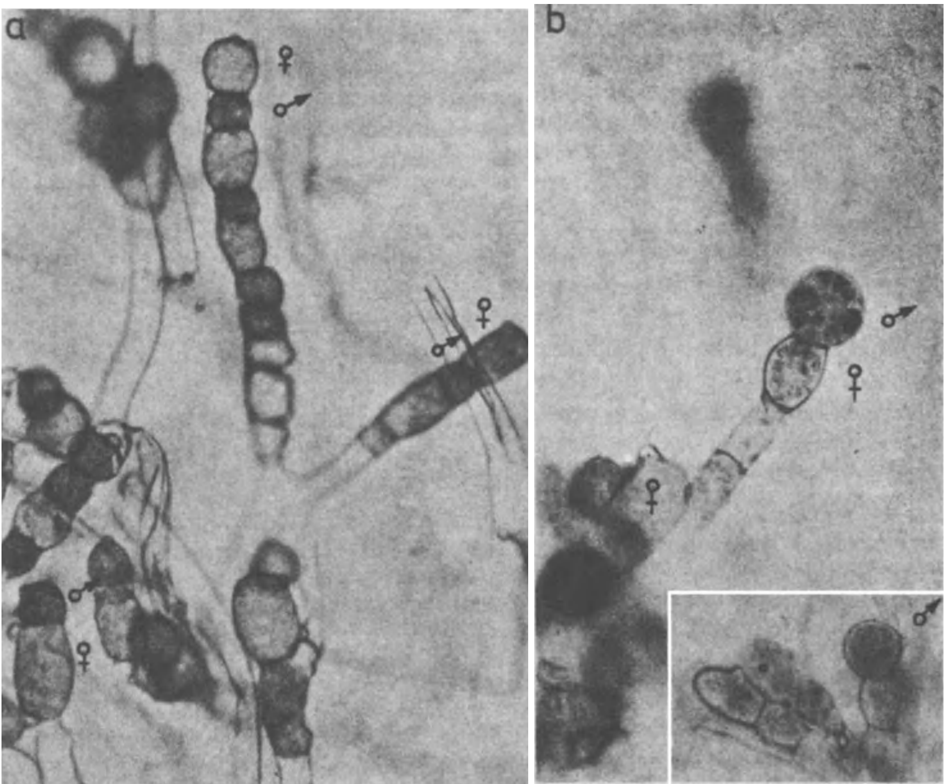

Fig. 2. (a) Gametophytic, epigynous *Allomyces macrogynus* (Strain Burma) originated from meiospores treated with DNA of hypogynous *A. arbusculus*. Note inverted polarity of *A. macrogynous* couples with dominance of the *A. arbusculus* gametangial shape. All small dark gametangia are male. × 600. (b) Inverse experiment as in (a). *A. arbusculus* as meiospore receptor for donor DNA of *A. macrogynus*. The dominance of shape of *A. arbusculus* is illustrated by the retention of receptor gametangial form, and the acceptance of the DNA-transferred character exhibited by the vivid yellow colour (dark in the picture) of the now male, epigynous gametangia. × 800 (inset × 600)

with DNA from *A. macrogynus*) was also successful. Treatment with homologous DNA and with calf-thymus DNA did not change the position of the gametangia. DNAase destroyed the transforming capacity of the DNA preparations. Unfortunately, no data are available regarding the maintenance of the new characteristics in subsequent generations (OJHA and TURIAN, 1971).

Such data, however, seem to be necessary. This was demonstrated by MISHRA *et al.* (1972) working with another fungus, *Neurospora*. Mutants that were defective in the synthesis of inositol (inos$^-$) or pyridoxine (pdx$^-$) or that showed a deviating colonial morphology (rg$^-$) were treated with the DNA of normal strains. Following this, revertants could be selected. In the best-studied inos$^-$ mutants their frequency was 0.95 per million. Treatment with inos$^-$-DNA resulted in only 0.03 revertants per million. Citrate buffer had the same negligible effect. DNAase abolished the transforming activity of inos$^+$-DNA. The new trait (inos$^+$) was maintained during vegetative growth, but most of the revertants lost the inos$^+$ character during meiosis or transmitted it into the progeny in a non-mendelian way. It was concluded that the transferred genetic material could exist in the receptors in the form of exosomes that might be eliminated during meiosis or transmitted into the progeny independently from the chromosomes (MISHRA and TATUM, 1973). The exosome model, based on experiments with *Drosophila* and modified according to our data from petunia will be discussed later (Sect. 5.2.3).

The most interesting data on gene transfer into cells in culture came from the laboratory of DOY *et al.* (1973a, b, c). The Australian group used the advantages of phage systems demonstrated by MERRIL *et al.* (1971, 1972) in human fibroblasts. Receptors were haploid tissue cultures of *Arabidopsis thaliana* and *Lycopersicum.* It should be mentioned that the haploid nature of the plant material in this and other cases is not essential for the expression of the exogenous gene material, but might be advantageous in producing diploid pure lines by polyploidization of modified cells.

The recipients in most of the experiments were calli of tomato. Normally, they die or grow very slowly on media containing galactose (2%) or lactose (4 or 10%) as carbon source. Following a treatment with phage material carrying the bacterial genes for galactose-1-phosphate uridyl transferase (λpgal$^+$), normal growth was induced in some calli on galactose media; the same was true on lactose media following a treatment with phages carrying the bacterial gene for β-galactosidase ($\emptyset$80plac$^+$). Control experiments with λpgal$^-$ in the case of galactose media and $\emptyset$80 in the case of lactose media were without any effect (Fig. 3). Controls with tomato DNA were not carried out. On lactose media a mixed infection with $\emptyset$80plac$^+$ and λpgal$^+$ was more effective than a treatment with only λpgal$^+$. This could be understood because in this case not only the glucose set free by the β-galactosidase activity but also the second splitting product of lactose, the galactose could be utilized.

In the $\emptyset$80plac$^+$ experiments the activity of β-galactosidase in the calli growing on lactose media was higher than in the controls. Due to unknown reasons the activity showed oscillations and declined gradually (DOY *et al.*, 1973a, b, c). It could be demonstrated that this activity was due to *E. coli* β-galactosidase, because the enzyme was protected by *E. coli* β-galactosidase antibodies against heat

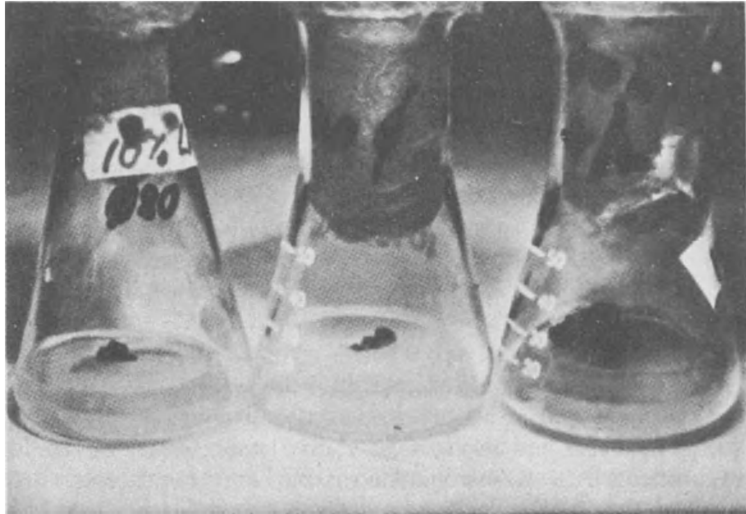

Fig. 3. Gene transfer by phage Ø 80plac$^+$ in tomato tissue cultures. The tissue was kept on lactose agar. From left to right: tissue treated with Ø 80plac$^+$, untreated control, control treated with Ø 80. Courtesy of Dr. P. M. GRESSHOFF

inactivation. The plant enzyme was heat denatured. It should be mentioned that bacterial contaminations could not be detected.

Independently, JOHNSON *et al.* (1973), working with λplac5 and *Acer pseudoplatanus* cells in suspension culture, obtained comparable results. Infection with λplac5 stimulated growth in lactose media compared with controls treated with λ^+ phages, not carrying the β-galactosidase gene. In this system, however, efforts to affirm the bacterial nature of the β-galactosidase by the immunological test just mentioned failed.

One source of possible error in these experiments, namely bacterial contaminations, can be excluded. Another difficulty could be that the plant material itself shows β-galactosidase activity, which for instance was detected in protoplasts, calli, seedlings and leaves of *Petunia* (HESS, 1976a), in flower buds and leaves of *Nemesia* and in cell suspension cultures of *Pharbitis nil* (MESSERSCHMIDT, in prep.). Also the control calli of *Lycopersicum* and the control cell suspensions of sycamore exhibited low β-galactosidase activity.

This activity must not necessarily be due to a special β-galactosidase but could result from other glycosidases with low substrate specificity. In our experience with petunia this β-galactosidase activity could vary greatly from plant to plant. Also DOY *et al.* (1973a, b, c) detected 5–10% of calli growing better than usual even on 10% lactose media. These calli, following the immunological assay, showed only the plant β-galactosidase activity. Further data are not given, but in view of the long duration of the experiments it seems highly probable that the better growth is due not to stored carbohydrates but to a higher β-galactosidase activity. So it seems necessary to include statistical evaluations in further experiments. In the meantime, one has to trust the reliability of the immunological assay. Here doubts could arise from the fact that impure plant materials were used.

Interesting results were also obtained by infecting the tomato cell cultures with ∅80supF$^+$, a phage carrying the *E. coli* mutant suppressor gene supF$^+$ (DOY *et al.*, 1973a, b). Kept on a glucose medium optimal for growth, the calli treated with ∅80supF$^+$ died while ∅80 controls grew normally. The molecular basis of this effect is unknown and the biological phenomenon is unusual. To induce callus death, one has to add 10^2 phages per plant cell. Despite this numerical superiority of the phages it does not seem likely that all the cells were "transformed to death". In this case, however, one would expect the living cells to overgrow the dead ones so that after some time normal callus growth would be restored. An explanation for the death of the callus could be the diffusion of a toxic product from the dead to the living cells. Whatever the interpretation of these phenomena might be, they demonstrate that in genetic manipulation of higher plants undesirable effects must be taken into consideration.

Sycamore cells in culture do not regenerate whole plants, and unfortunately, the tomato cells had also lost the ability to regenerate. So one should repeat the experiments with another system capable of regeneration in vitro. Apart from the confirmation of the results discussed here, data are needed for the long-term maintenence of the transferred gene material, and through the barrier of meiotic divisions.

Differentiation might be the catchword for discussing another system. Normally, calli can be comparatively easily induced to regenerate roots, but difficulties often arise in inducing shoots. Rooting of shoots, however, usually offers no problems under sterile conditions. It is also possible to regenerate whole plants from isolated apical meristems. One only has to remember the clonal multiplication of orchids by apical meristems, a method widely used in practice (MOREL, 1974). In overcoming possible difficulties in shoot differentiation and in preserving the advantages of sterile work with tissues in culture, it is tempting to use apical meristems or shoot tips as receptors of foreign genetic material. HOLL *et al.* (1974) used shoot tips of a cultivar of *Pisum arvense* which neither formed nodules nor fixed nitrogen. They were treated with the DNA of another *Pisum* variety which formed both. 1–2% of the plants regenerated from the shoot tips appeared to be corrected for both characteristics. This double correction seems to be highly improbable, except when the genes governing them are closely linked. Data about the genetics are not given, but the authors themselves point out that extreme precaution in the interpretation of their results is necessery. In any case some critical control experiments are missing, for instance treatment of the double mutant with their own DNA.

5. Cells in Plants, Seedlings and Embryos as Receptors

5.1 Uptake

Let us start with a remark on DNA uptake by vegetative parts of adult plants because these studies have only remote relation to genetic manipulations. The first report of this kind, dealing with DNA uptake into tomato shoots, was jointly

published by STROUN and LEDOUX and their coworkers (STROUN *et al.*, 1966), and was continued by STROUN and ANKER. Most of these publications are cited in a more recent review (HESS, 1972a), so that a short discussion taking into consideration more recent findings in other systems will be sufficient. After dipping the tomato shoots into solutions with labeled bacterial DNA, and after removing the plant part brought into contact with these solutions, labeled bacterial DNA could be reextracted in an apparently slightly degraded state.

Plating tests on bacteria remained negative. Furthermore, control plants treated with ^{3}H-thymidine showed only one labeled peak with the buoyant density of Solanum DNA (ANKER and STROUN, 1968); so bacterial contaminations seem unlikely.

The next question was whether the reextracted DNA was taken up into the living cells, especially into the nuclei. It seemed possible that the DNA was located only in the dead xylem elements, and that the nuclei showed radioactivity (STROUN *et al.*, 1966) due to the re-utilization of breakdown products. To exclude this possibility, HOTTA and STERN (1971) isolated the nuclei from tomato shoots fed with ^{32}P-*Micrococcus lysodeicticus* DNA. They found bacterial DNA in the nuclear fraction but the exact localization of this DNA before the isolation of the nuclei could not be determined. It was concluded that the bacterial DNA might have been present either in the cytoplasm or adsorbed to the nuclei. There was no indication for an uptake into the nuclei themselves. Perhaps one could even go "back" one step more in arguing that the bacterial DNA could have been caught from any location outside the cells during the destruction of the tissue in the course of the isolation of the nuclei (cf. Sect. 3.1.1).

There is a claim that tobacco leaves inoculated with the DNA of bacteriophage fd could produce complete phage particles (SANDER, 1964) and that the host specificity of the phages could be altered by the passage through the plant cells (SANDER, 1967). However, there is no convincing evidence that these effects are due to the plant cells and not to bacteria associated with them.

Let us now turn to seeds and seedlings. Most of the work comes from the laboratory of LEDOUX and was done with *Hordeum* or *Arabidopsis*. These data are important for obtaining genetically altered plants.

In the case of barley (LEDOUX, 1965; LEDOUX and HUART, 1969), seeds were sectioned at one end and brought into the DNA solutions under investigation. Using CsCl density-gradient analysis 3H-labeled bacterial DNA already fed could be reextracted from the scutellum and the cotyledons. In the coleoptile the exogenous DNA was degraded. Here once more one wonders whether bacterial contaminations could be involved.

In the much more intensive *Arabidopsis* experiments, carried out under aseptic conditions (LEDOUX *et al.*, 1971a, b; 1972), seeds were soaked with ^{3}H-labeled bacterial DNA and grown. In the beginning the media consisted of perlite soaked with the nutrient solution. Later on agar media were used. The latter medium seems to be preferable for optical detection of bacterial contaminations in long-term cultures.

There is no statement that biological tests for bacterial contaminations were carried out, but control experiments with ^{3}H-thymidine were included in all important experiments. From plantlets developed from seeds treated with ^{3}H-labeled DNA of different bacteria, ^{3}H-labeled DNA with the buoyant density of

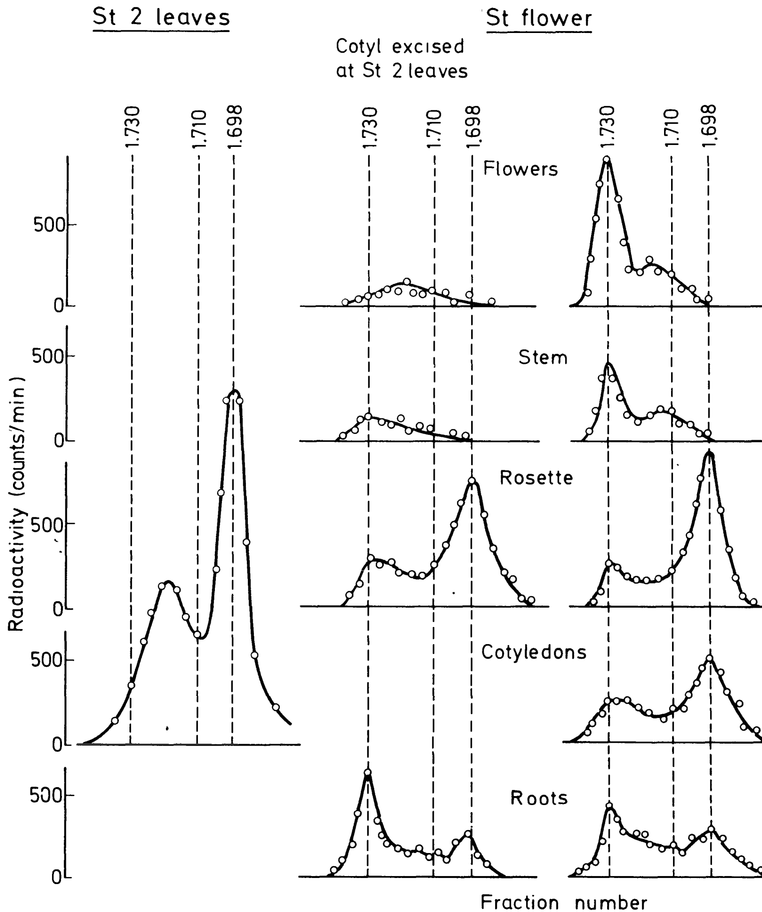

Fig. 4. Translocation of stored bacterial DNA in *Arabidopsis* at the stage of flowering. CsCl density gradient centrifugation of DNA isolated from different organs of groups of 80 plants grown from seeds treated for 4 days with *Streptomyces coelicolor* 3H-DNA. Buoyant densities (g/ml): 1.698 *Arabidopsis* DNA; 1.710 *E. coli* DNA; 1.730 *S. coelicolor* DNA. *Left* DNA profile at the two-leaf stage *(St 2 leaves)*. There are two DNA peaks, the *Arabidopsis* DNA peak and an "intermediary" peak (cf. text). If the cotyledons are excised at this two-leaf stage, then at the flower stage no translocation of bacterial DNA into the flowers can be found *(St Flower, left half)*. If the cotyledons were not excised, then there appears a DNA peak with the buoyant density of *S. coelicolor* DNA in the flowers and stems *(St flower, right half)*. Bacterial contaminations seem to be excluded because in this case one also expected a *S. coelicolor* DNA peak after excision of the cotyledons. (From Ledoux *et al.*, 1972)

the bacterial DNA used could be reisolated. In experiments using ^{3}H-thymidine no DNA of this kind could be detected. Also the plant DNA may be labeled, indicating a partial breakdown and re-utilization of the foreign DNA.

Two further findings deserve to be discussed. First, a translocation of bacterial DNA stored in the cotyledons to the flowers could be demonstrated (Fig. 4). Once more controls with ^{3}H-thymidine excluded bacterial contaminations (Ledoux *et al.*, 1971 b).

Considering translocation of DNA one should not forget that the first report of this kind was given by STROUN *et al.* (1967b) working with *Solanum lycopersicum*. Control experiments with ^{3}H-thymidine were included as well. The STROUN group later studied the influence of several factors on translocation (light: STROUN *et al.*, 1967c; temperature: STROUN *et al.*, 1968; citrate: ANKER *et al.*, 1968). A translocation of bacterial DNA seems to take place in *Sinapis alba* (LEDOUX *et al.*, 1975). In this case, however, it was not possible to maintain sterile conditions.

A "circulating DNA", perhaps with messenger function, would be of importance not only in plant but also in animal systems (ANKER and STROUN, 1972). As far as genetic manipulations are concerned, one might speculate that exogenous DNA could be carried into the target cells not only at the time of feeding, but even much later, translocated from a DNA pool.

Secondly, in several experiments the groups of STROUN (ANKER and STROUN, 1968) and LEDOUX (LEDOUX *et al.*, 1971 a, b) detected, after feeding labeled bacterial DNA, a peak intermediate in buoyant density between the plant's own DNA and the bacterial DNA fed. LEDOUX *et al.* (1971 a, b) analyzed these "intermediary" peaks more intensively and came to the conclusion that it should be bacterial DNA covalently linked end to end to the plant DNA. So far, these intermediary peaks seemed to be the first biochemical evidence for an integration of exogenous DNA. In repeating some of LEDOUX's experiments, HOTTA and STERN (1971) found the intermediary peaks as well, but only under abnormal physiological conditions (desiccation, strong irradiation). Furthermore, they came to the conclusion that the exogenous DNA was bound only to the surface of the nuclei (cf. Sect. 5.1), so that a "hybrid" formation under physiological conditions was apparently not possible. The publication of HOTTA and STERN (1971) was often interpreted as a refutation of the work of LEDOUX's group. One should not overlook, however, the fact that the results obtained by both groups are essentially the same (with the exception that the intermediary peaks did not seem to indicate an integration in the latter case). Recently, KLEINHOFS (1975), studying the fate of exogenous DNA by DNA hybridization techniques, claimed that the intermediary peaks might be due to bacterial contaminations. In any case, clarification of these controversial findings is urgently needed.

BENDICH and FILNER (1971) demonstrated that pea seedlings could take up *Pseudomonas aeruginosa* DNA through their roots. The bacterial DNA reextracted was partially degraded. Following their interpretation there was no proof that foreign DNA was taken up into the nuclei. REBEL *et al.* (1973) worked with ^{32}P labeled T4 phage DNA and *Matthiola* seedlings. They concluded from their data that the T4-DNA was taken up by the seedlings and transformed into a double-stranded fraction with higher buoyant density. Unfortunately, the density of the T4-DNA (1.694) lies in the range of the density of the *Matthiola* DNA (1.698). Furthermore, there is a plant satellite DNA of higher density that should overlap the T4 DNA. In some experiments the time of CsCl density gradient centrifugation seems rather low (24 h). So one should await further clarification.

Let us summarize: If cut grains of barley (LEDOUX, 1965), or tomato shoots without roots (ANKER and STROUN, 1968; HOTTA and STERN, 1971) were brought into contact with the DNA solutions it is impossible, without additional data, to decide whether the reextracted DNA was located in intercellular spaces or in dead vessels or in fact taken up into living cells. The isolation of the nuclei (HOTTA and STERN, 1971) also does not help here because, during their preparation, the nuclei may have absorbed DNA initially located outside the living cells. HOTTA and

Stern (1973) themselves demonstrated that the membranes of isolated nuclei may adsorb DNA.

In the case of seeds *(Arabidopsis)* and seedlings *(Pisum, Matthiola)*, however, there is no artificial opening in the cell cover so that one could assume that the DNA was indeed taken up. A translocation seems to be possible. In most cases the DNA was partially degraded. An uptake of the exogenous DNA into the nuclei and its integration into the host's DNA can not yet be proven, so we are at the same point as in the protoplast work.

5.2 Expression

As discussed in the preceding section, swelling seeds and young seedlings of several plant species may take up exogenous DNA in a sometimes partially degraded, but nevertheless, high molecular weight state. The potential for a successful gene transfer thus seems to be present. The chances for success seem to be higher in the correction of mutations than in the introduction of a completely new character, because in the former case even a short segment of DNA—just long enough to cover the mutated DNA segment and to allow the different polymerases to work—would be sufficient. Degradations could therefore even be useful in enabling a better penetration of the shortened DNA molecules. In fact, most of the positive findings reported so far deal with the correction of mutations.

5.2.1 *Petunia* Experiments

In *P. hybrida* a transfer of genetic material from one pure line to the other for anthocyanin synthesis and leaf shape was apparently obtained by treating ovaries (at the time of fertilization) (Hess, 1973 b), seeds (Hess, 1969 b) or seedlings (Hess, 1969 a, b; 1970 a, b; 1971 a) with the appropriate DNA.

It is essential in such experiments that true-breeding pure lines and not commercial varieties be used. The genetics of the pure lines used in the petunia experiments are well known, for they were for many years objects of investigations on the biochemical genetics of anthocyanin synthesis (Hess, 1968).

5.2.1.1 Anthocyanin Synthesis

Treatment of a white-flowering pure line with the DNA of a red-flowering, anthocyanin-synthesizing pure line resulted in a genetically stable correction for anthocyanin synthesis in 0.06% of the treated plants (Fig. 5). Most of the corrected plants were unexpectedly homozygous (Hess, 1970 a), but heterozygous and chimeric plants could also be found (Hess, 1972 b). The transferred gene material, normally dominant, showed in the receptor a recessive behavior. The probably transferred gene material was normally located on the chromosomes carrying the mutant locus. But in one case, after the DNA treatment, there was a gene-inducing anthocyanin synthesis on a chromosome that previously had carried no gene of this kind, neither normal nor mutated. This finding would be best explained by a successful gene transfer. Controls with buffer or with homologous DNA were negative. DNAase destroyed the transforming activity (Hess, 1971a).

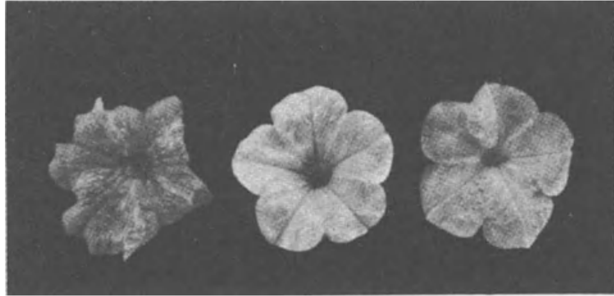

Fig. 5. Transfer of gene material for anthocyanin synthesis in *Petunia*. From left to right flowers of the red-flowering DNA donor, the white flowering DNA receptor and of one homozygous corrected plant. (From HESS, 1971a)

The experiments were extended over five years. During this time 5 out of 25 major experiments were positive. To keep this in perspective, it must be mentioned that over a period of more than 1.5 years all experiments had been unsuccessful. Whether or not the gene transfer was actually successful has been widely debated, including many critical comments:

a) The number of corrected plants seemed rather high (e.g. BIANCHI and WALET-FOEDERER, 1974). In fact, the first experimental series was somewhat obscured by a slight pigmentation in a high percentage of experimental as well as control plants. The pigmentation was partially due to insects, and partially perhaps to viral infections. Usually viruses block anthocyanin pigmentation, but there are also some reports on stimulation of anthocyanin synthesis (RADEMACHER und SCHWARZ, 1958; FEENSTRA *et al.*, 1963). The nutritional conditions may also play a part. The biochemical and genetical background for such anthocyanin synthesis observed in several species under more or less non-physiological or pathological conditions is unknown.

In any case, this pigmentation was usually very weak, visible only in the flower buds, and disappeared during the elongation of the petals. In all cases tested by selfing or outcrossing this slightly red pigmentation proved to be genetically unstable. The plants just discussed could be easily differentiated from the apparently corrected plants by their comparatively high anthocyanin content. Although we never claimed this, they were counted by several colleagues as corrected plants resulting in an artificially high number of corrected plants. The percentage of corrected plants, on the contrary, is even lower than in the *Arabidopsis* experiments (0.06 versus 0.19 to 0.89%, LEDOUX *et al.*, 1974) or in bacterial transformations.

b) Virus infection. It has just been mentioned that in rare cases a virus infection could stimulate anthocyanin synthesis. But our apparently corrected plants showed Mendelian segregation for anthocyanin synthesis, a fact that cannot be explained with a virus infection (HESS, 1970 a).

c) Homozygous corrected plants. In experiments on the correction of organisms by gene transfer (*Petunia*: HESS, 1970a; *Arabidopsis*: LEDOUX *et al.*, 1975; for animal

systems cf. literature in HESS, 1972 a) homozygous corrected plants were detected. If one envisages the difficulties connected with the correction process one could be more than content if the exogenous material was integrated in one of the two homologous chromosomes in one of the initial cells.

Therefore, one would expect, (1) phenotypic mosaics—provided that the gene products under question cannot diffuse from cell to cell, and (2) a heterozygous state. Chimeric and heterozygous plants were found (HESS, 1972 b), but the appearance of homozygotes remained a problem, as repeatedly pointed out while discussing the petunia results (HESS, 1970a, b; 1972a, b).

BIANCHI and WALET-FOEDERER (1974) years later, while studying the apex morphology of petunia seedlings, realized this difficulty as well. First, they concluded from observations with flower color chimeras of *Petunia* that "dominant alleles of anthocyanin synthesis could only express themselves in those cells in which they are present". This generalization is not correct, for years before HESS (1963) had demonstrated, in grafting experiments with red and white flowering petunia lines, that there was no influence from one grafting partner to the other on anthocyanin synthesis. But this holds true only for long distances. Experiments using corn endosperm with different genetic compositions and periclinal chimeras of *Euphorbia pulcherrima* had demonstrated that there might well be an influence over short distances, so that in genetically defective cells anthocyanin synthesis was restored (for literature see HESS, 1968). It depends upon the kind of the genetic difference, e.g. from the diffusible or nondiffusible character of the relevant gene product, whether such an interaction can take place. So even this first statement deserves further analysis.

In extending their arguments BIANCHI and WALET-FOEDERER (1974) claimed that somatic mutations at the seedling stage, caused by treatment with exogenous DNA, would induce chimeras in flower pigmentation. After pointing out that we had not found chimeras of the kind they expected, and that we had not taken into account the existing differentiation of the shoot apex in interpreting our results, they concluded that all our data could be explained if one assumed a replication of the exogenous DNA in the receptor organism.

In voicing this criticism, BIANCHI and WALET-FOEDERER (1974) overlooked the fact that we treated not only the seedling stage studied by them but also much earlier developmental stages (seeds and ovaries, cf. above). It should be noted that most of these points were discussed (HESS, 1972 a) two years before the publication of BIANCHI and WALET-FOEDERER (1974). HESS (1972a) pointed out that the existence of completely corrected and homozygous plants could hardly be explained other than by a replication of the exogenous DNA, just as BIANCHI and WALET-FOEDERER later postulated.

d) Somatic mutations. Apparently the publication of BIANCHI and WALET-FOEDERER (1974) has sometimes been misinterpreted as HEYN *et al.* (1974) claimed that in the investigations of BIANCHI and WALET-FOEDERER "by spontaneous (somatic) mutations ... mosaic flowers are produced with a frequency in the same range which has been claimed by HESS (1972 a, b) to occur in DNA-treated plants". Here one has to defend BIANCHI and WALET-FOEDERER. They did not attempt to explain the petunia experiments on the basis of somatic mutations.

Furthermore, there are no data for the frequency of the somatic mutation events in their publication, however, this misunderstanding could cause one to ask whether somatic mutations could be involved. First of all, these hypothetical mutational events were not observed following treatments with control DNA, e.g. the DNA of the white flowering line itself. Besides that one should mention that in flower color genetics, mutations from red to white are the rule. Also BIANCHI and WALET-FOEDERER (1974) were dealing with a somatic mutation from red to a weaker red. The reverse case, mutation from white to red, should be expected, for instance, in the very rare cases of a dominant white (e.g. in *Nemesia:* HESS, 1969 c, 1971 b)

Several other possible interpretations (permanent derepression of anthocyanin genes, reverse mutation, „dauer“ modification) were also discussed and ruled out (HESS, 1970a). For leave's shape see Sect. 5.2.3.

5.2.2 *Arabidopsis* Experiments

During and following their uptake studies LEDOUX and his coworkers investigated biological effects of the transferred bacterial DNA. *Arabidopsis* with its nutritional mutants, small size and short life cycle seems to be best suited for studies of this kind. Some of the first experiments seem to be less substantiated, probably due to possible bacterial contaminations and to a leaky character of the mutants used. But more recently, convincing evidence was obtained that thiamine-less *Arabidopsis* mutants could be corrected by exogenous DNA (LEDOUX *et al.*, 1974, 1975).

Seeds of the mutants were treated with bacterial DNA of different origins carrying the gene material for thiamine synthesis and in the controls, with the DNA of a thiamine-less *E.coli* mutant, with phage DNA not carrying the bacterial genes for thiamine synthesis, and with buffer. The seeds soaked with the DNA solutions were tested on thiamine-free agar media (in some cases similar to earlier tests on perlite). The different thiamine, thiazole and pyrimidine-requiring mutants were corrected in a changing percentage. *E.coli* DNA (0.89% corrections) turned out to be the most effective, and *Streptomyces coelicolor* DNA (0.19% corrections) the least. In the controls no corrections could be observed. All the corrected plants, growing in the absence of thiamine, showed a delay in development compared with the wild type or with mutants supplemented with thiamine. So in this case too, the expression of the probably transferred gene material is less than normal.

By selfing and outcrossing, it was found that the corrected plants behaved like true breeding homozygotes, which is another parallel to the petunia experiments. The results of reciprocal crosses between corrected and uncorrected mutants exclude the possibility of a maternal inheritance. The F 1 to F 3 plants originating from corrected mutants in most cases showed a variegated pattern of chlorophyll pigmentation. As in the case of the parental generation, their growth was less than that of mutants supplemented with thiamine, or of the wild strain. Furthermore, segregation was observed in the progeny of corrected mutant outcrosses with the wild type. A complete range of phenotypes, from corrected to incompletely corrected, and the uncorrected lethal mutant phenotypes was found. As in *Droso-*

phila (FOX *et al.*, 1971), *Petunia* (HESS, 1972 a, b) and in *Neurospora* (MISHRA and TATUM, 1973) LEDOUX also interpreted these results in terms of the exosome model discussed below. If the work is critically evaluated, one is tempted to find more explanations than corrections by exogenous DNA. But if the main sources of error, bacterial contamination and leaky mutants are excluded, there are only a few points that deserve further mention.

First, the mutants were never treated with homologous mutant DNA or with the DNA of the wild type of *Arabidopsis*. According to LEDOUX *et al.* (1974), this is due to the impossibility of preparing intact high molecular weight DNA from *Arabidopsis*, which he considers as a prerequisite for efficient uptake and integration. One might doubt whether, for instance, the uptake should be alleviated if the DNA molecules are larger. But apart from this, it would be interesting to know whether homologous *Arabidopsis* DNA, partially degraded or not, would exert any effect.

Second, one should try to prove that thiamine and its precursors are synthesized by bacterial enzyme systems in the corrected mutants. No doubt this may be easier to say than to do. Third, one may find it difficult to explain the phenotypic mosaicism found as the base of LEDOUX's interpretation. If the thiamine-less mutants used in the work of LEDOUX are supplemented by thiamine, they show normal appearance or at least no mosaicism (LEDOUX *et al.*, 1974, 1975). The vitamins, or in other mutants their precursors, are taken up by the root system, translocated, and exert their influence all over the plant. [This is in contrast to the situation in anthocyanin synthesis discussed earlier where no influence from one graft partner to the other could be found (HESS, 1963).] Therefore, in the case of the corrected mutants one should expect that the cells corrected for thiamine synthesis would supplement their neighbor cells that were not corrected, that lacked transcription of the exosome or that had lost the exosome again.

The phenotype expected should be uniform. As for the generation treated one should take into consideration that other DNA with harmful effects may be taken up into some cells. But difficulties arise in extending this explanation to the offspring.

Last but not least, the parallelism of the results obtained from such different systems as *Petunia* and *Arabidopsis* (e.g. weak phenotypic expression of the transferred gene material, appearance of homozygous corrected plants in the treated generation, phenotypic mosaicism in the corrected plants (evidence for petunia below) should be stressed (cf. HESS, 1972 a), although, the sceptic would argue that a double impossibility would still be an impossibility.

5.2.3 The Exosome Model

As mentioned earlier, the exosome model created by FOX *et al.* (1971) was modified to explain the results obtained with *Petunia* (HESS 1972 a, b; 1973 b), and later on served as a basis for discussion of the results obtained with *Neurospora* (MISHRA and TATUM, 1973) and *Arabidopsis* (LEDOUX *et al.*, 1975). The model may be introduced in the "Petunia"-version (Fig. 6), because in the *Petunia* experiments rather convincing evidence for the realization of this model was obtained. An exosome is defined as a gene carrier, e.g. DNA, that might be located in the

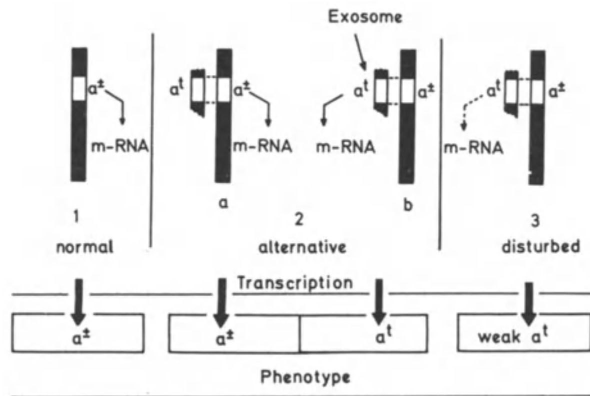

Fig. 6. The exosome model (HESS, 1972a). *1* normal transcription of allele $a^{\pm}$. *2* alternative transcription of either the normal allele $a^{\pm}$ or—in other cells—of the exosome a^{t} will result in a mosaic phenotype $a^{\pm}/a^{t}$. *3* disturbed transcription leads to a weak expressivity. Only one of the two homologous chromosomes is shown

cytoplasm or might change its location from the cytoplasm to the chromosomes. In contrast to the episome, it may be closely associated with the chromosome, but it is never integrated into its linear structure. The exosome may be fixed anywhere on the chromosome, but preferentially to complementary nucleotide sequences, provided that they are present in the host genome. The exosome may be transferred into the cytoplasm again, and so could be maternally inherited as discussed for *Neurospora* (MISHRA and TATUM, 1973). Alternatively, the exosome set free from the chromosomes could be lost as discussed for *Arabidopsis* (LEDOUX *et al.*, 1974). If it remains fixed to the chromosomes over the meiotic events one will find a Mendelian inheritance as demonstrated in *Petunia* (HESS, 1970a).

Now to the phenotypic expression of the exosome fixed to its chromosome (if the polymerases were present, an expression in the cytoplasm could take place as well). Three alternatives are possible: (1) The exosome is normally transcribed. In this case one would find a normal phenotypic expression of the transferred gene material. (2) The RNA polymerases may have difficulties in transcribing the exosome as it is not integrated into the linear structure of the chromosome; the transcription rate is thus lowered, and the phenotypic expression is weaker. (3) The exosome is not transcribed at all. As the internal conditions may vary from cell to cell, it seems possible that in one cell a transcription takes place, while not in another. The result of this alternative transcription would be a phenotypic mosaicism as was found in *Arabidopsis* (but cf. discussion of the *Arabidopsis* mosaicism in Sect. 5.2.2) and in *Petunia* (HESS, 1973 b).

The results obtained in *Petunia* are consistent with the main features of the model: there is a weaker phenotypic expression of the transferred gene material as indicated by its change from dominant to recessive behavior (HESS, 1970 a, b). In plants heterozygous for a transplanted gene of anthocyanin synthesis one detects anthocyanin pigmentation in fully expanded flowers only along the veins leaving the corollar tube. Plants homozygous for a transplanted gene of anthocyanin synthesis also show a much weaker pigmentation than normal—pink instead of

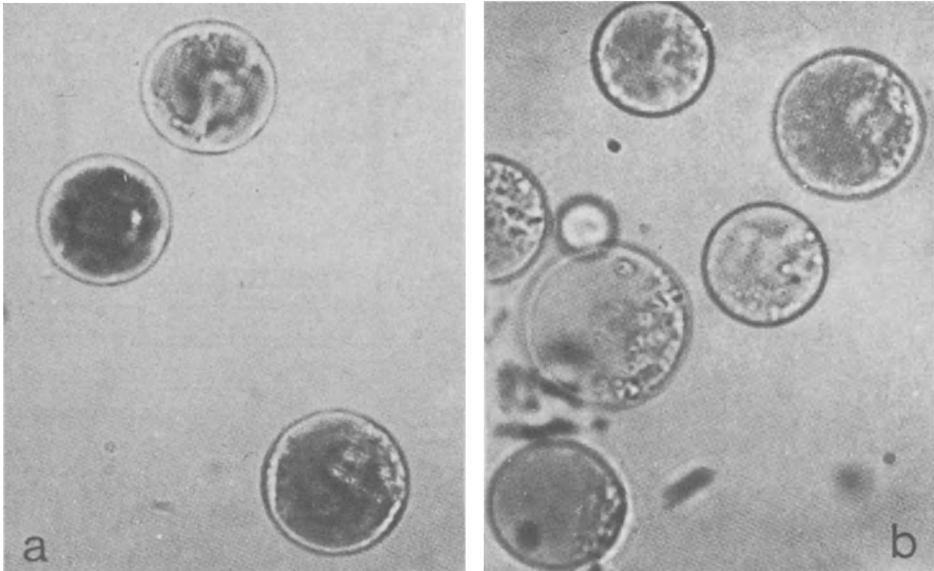

Fig. 7a and b. Transfer of gene material for anthocyanin synthesis. Protoplasts isolated from the petals of the DNA donor (a) and from the petals of a plant homozygous corrected for anthocyanin synthesis (b) 320 ×. (From HESS, 1973b)

deep red. Cytological observations showed that the pink color was due partly to a phenotypic mosaicism brought about by a mixture of epidermal cells containing anthocyanins with anthocyanin-free ones. Furthermore, the anthocyanin-containing cells also showed a lower anthocyanin accumulation than normal as proven by microphotometric measurements in protoplasts isolated from the petals (Fig. 7; HESS, 1973 b). So in the corrected *Petunia* flowers there is a phenotypic mosaicism as well as a weak phenotypic expression of the probably transferred gene material. The central point in the exosome model seems to be that two genetic informations for a given characteristic, i.e. the information of the DNA receptor and the introduced foreign information, could be present. If one could prove this double genetic base, both the exosome model and the gene transfer itself would be much more probable. Of course, this proof will be practicable only if both sets of information could be expressed phenotypically. In this connection some petunia experiments will be discussed. The pure lines of petunia used in our anthocyanin experiments differ not only in pigment synthesis, but also in leaf shape. The red flowering form has round, wrinkled leaves; the white flowering form has narrow, flat leaves. Leaf shape is governed by one gene locus, the allele governing round leaves being incompletely dominant over the allele inducing narrow leaves. Young hybrid plants show narrow leaves with only a slight tendency toward roundness. During development the leaf form changes so that adult plants show the round leaf form with a tendency toward narrowness (HESS, 1970 b): that means both alleles are physiologically active—a prerequisite mentioned above.

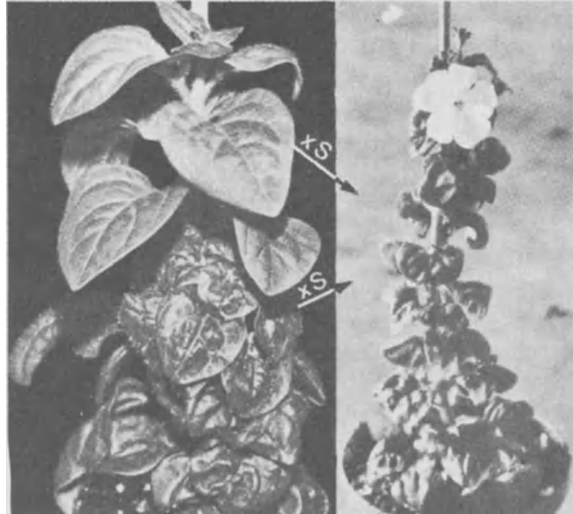

Fig. 8. Proof for two kinds of genetics informations of a given characteristic, leaf shape. *Petunia* seedlings with the gene material for narrow leaves were treated with the DNA of a pure line with round leaves. In the progenies plants could be obtained that were homozygous for the transplanted allele for round leaves. They show the round leaf shape. In ca. 1% of these round leafy homozygotes the narrow leaf form of the DNA receptor reappears during development *(left)*. By selfing flowers on shoots with the narrow as well as on shoots with the round-leaf shape one obtains uniform progenies with the round-leaf shape *(right)*. The experiment demonstrates that following DNA treatment two different alleles, for narrow as well as for round-leaf form may be present in the same plant parts. (From HESS, 1972a)

Seeds (HESS, 1970b) or ovaries (HESS, 1973b) of the pure line with narrow leaves were treated with DNA of the round leaf form. The treated generation show uniformly narrow leaves, but, by selfing them repeatedly (HESS, 1972a, 1973b), plants with round leaves could be detected. They proved homozygous for the allele governing round leaf form, and are derived from the respective heterozygotes produced by the DNA treatment.

In about 1% of these round leafy homozygotes the narrow leaf form of the DNA receptor reappears during development, either in parts of the leaves or on axillary shoots or even on the older main shoot. This effect could be explained only if the alleles both for round leaves and for narrow leaves were present in these plants. The question was whether the gene for round leaves was lost or still present in an inactive state in the plant parts that had changed to the narrow character. Also the leafy organs of the flowers on narrow-leaf twigs show the "narrow" shape. It was thus possible to self flowers with the narrow characteristic. The progeny at first showed uniformly round leaves (Fig. 8). As development progressed, ca. 1% of the upgrowing plants showed the same change from round to narrow leaves found in their parents. So in the narrow leafy shoots the allele-inducing round-leaf form was present as before. The best interpretation of these findings seems to be that in the plants transformed for round-leaf form, the alleles for round as well as for narrow leaf were maintained. Depending on inner condi-

tions which change during development, sometimes one and at other times the other may be transcribed. This is exactly what one has to postulate if one accepts the exosome model.

5.2.4 Replication

In *Petunia* the gene material for anthocyanin synthesis remained stable over six sexual generations tested. Thereafter the plants were propagated by cuttings for several years and the anthocyanin synthesis also proved stable. In *Arabidopsis*, the corrected state was maintained for at least three generations (LEDOUX, 1975). These data are in favor of a replication of the exogenous gene material. On the other hand, there is little biochemical data on DNA replication. Tomato shoots (STROUN *et al.*, 1967 c) and *Arabidopsis* seeds (LEDOUX, 1971) were treated with unlabeled bacterial DNA. The tomato shoots were dipped for 48 h into unlabeled DNA of *Agrobacterium*. Then, the shoots were put into water for several intervals ranging from 24–120 h and, thereafter, for 12 h into a solution containing 3H-thymidine. Following this, a labeled DNA of a buoyant density intermediary between *Agrobacterium* and tomato DNA was found that could be sheared by ultrasonication into two fractions, one of them with the density of *Agrobacterium*, the other with the density of tomato DNA. Only a few bacteria could be detected in the assay. Furthermore, the intermediary peaks did not appear if the shoots were treated with tomato DNA or no DNA or 3H-thymidine. So the intermediary peaks could not be due to bacterial contaminations. Using comparable methods, LEDOUX *et al.* (1971 a) claimed to have found a replication of bacterial DNA even in the progenies of the *Arabidopsis* seeds treated with this DNA. Again "intermediary" peaks appeared. So, for the moment, the biochemical data on replication are not satisfying. One should not overlook, however, that STROUN *et al.* (1967 c) took all care to exclude the participation of bacteria.

6. Pollen as Vehicles for Exogenous Genetic Material

6.1 The Working Hypothesis

Two conclusions result from these discussions: (1) one should stay as close as possible to the natural development processes to minimize the difficulties in growing and testing large numbers of progeny; (2) one should introduce the foreign genetic material as early as possible to be sure that a completely or at least largely genetically altered organism would result. Both requirements would be fulfilled if one could introduce the exogenous genetic material by means of pollen. Our working hypothesis: Pollen is incubated and germinated in exogenous gene material. During the incubation this material is taken up or at least firmly fixed to the pollen. This pollen is used for pollination of plants of the same species. The exogenous genetic material, taken up or adhering, is transmitted with the growing pollen tubes through the stylar tissue. During fertilization it is brought into the egg cell or it is taken up later on from the surrounding tissues into the young

developing embryo. Seeds are obtained that could be tested for genetic changes on appropriate nutrient media (HESS, 1974, 1975 b).

There is an additional advantage if very early developmental stages of pollen are used in experiments for DNA uptake, because such stages not only regenerate haploid plants in anthers cultured on agar-medium, but are also able to grow into haploid plantlets after their isolation from the anther (NITSCH, 1974; REINERT *et al.*, 1975; for further details see Chap. II.1.2). The liquid culture medium offers the best conditions for DNA uptake at the early stages, and there is a real chance that genetically changed plants could be raised and diploidized. In addition it must be pointed out that our experiments used no young developmental stages but only older pollen which was either ready to germinate or nearly capable of germination (as required by our working hypothesis).

6.2 Uptake into Pollen

First experiments indicated that pollen of *Petunia hybrida* and *Nicotiana glauca* germinate well in DNA solutions up to a concentration of about 100 μg/ml. Following pollination with this material a reduced number of seeds was obtained. This seems due to the fact that the pollen was put onto the stigma as a suspension so that its quantity was lower than by a normal pollination with dry pollen.

One tenth of the known plant viruses can be transmitted through seeds, infected either by the mother plant or by the pollen (MATTHEWS, 1973). So the main question was whether the pollen would take up exogenous genetic material under experimental conditions, and not whether it would be transferred.

Experiments to transfer *Arabidopsis* mosaic virus and tobacco ring sport virus, both known to give visible symptoms on *Petunia*, by *Petunia* pollen incubated with the virus material, were inconclusive. First of all, *Petunia* is a recommended stock material for the maintenance of viruses so it is difficult to differentiate uncontrolled contaminations from other virus material. Second, there seems to be a low molecular weight factor in the styles of *Petunia* inactivating plant viruses.

In *N. glauca* there is some autoradiographic evidence consistent with an uptake of labeled bacterial DNA into pollen, but, as proof, these data are not sufficient (HESS *et al.*, 1974). Therefore, pollen was germinated in ^{3}H-labeled *Rhizobium* DNA, then washed and treated with DNAase to remove excess DNA. To reduce the possibility that DNAase-insensitive complexes could interfer (cf. above) a treatment with triton X-100 followed. Triton weakens the exine and intine; therefore, the bound DNA should be set free so that it can be removed by further washings. These procedures also eliminated the DNA wedged between exine and intine. Following extraction, *Rhizobium* DNA could be detected in the CsCl density gradient (Fig. 9). The same experiment was done with young petunia pollen which did not germinate during the incubation period. In this case, only traces of labeled *E. coli* DNA could be reextracted from the pollen. So germination seems to be a prerequisite for a more intensive uptake (HESS *et al.*, 1974 b).

Proteins are also taken up by pollen of *Petunia*. Following a treatment of the pollen with fluorescein isothiocyanate (FITC)-labeled bovine serum albumin the protein fraction extracted from the pollen contained, as revealed by polyacry-

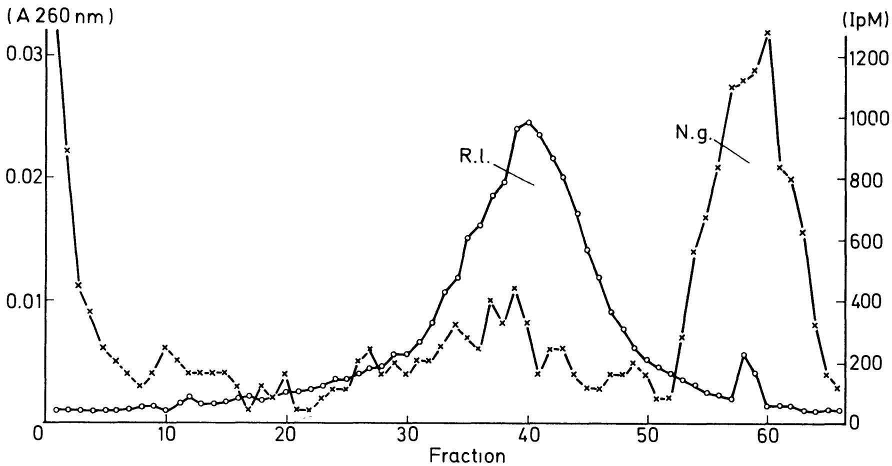

Fig. 9. CsCl gradient centrifugation of DNA isolated from germinating pollen grains of *Nicotiana glauca*. The pollen material was incubated with 3H-labeled DNA from *Rhizobium leguminosarum* as indicated in the text. ×–×–× : extinction 260 nm; ·—·—·—: radioactivity. *R. l.* = DNA of *R. leguminosarum*; *N. g.* = DNA of germinating *N. glauca* pollen grains. (HESS *et al.*, 1974b)

lamide gel electrophoresis, the serum components fed. An uptake of phage material could be demonstrated as well, using antibodies to inactivate any adhering phage material (HESS *et al.*, 1974a). Following pollination with petunia pollen incubated with phages λlac$^+$ plaques forming units could be extracted from the ovaries of the pollinated plants—in low yield, however, and only for a short time (HESS, 1976 b).

6.3 Expression

6.3.1 DNA

Hybrids from *N. glauca* and *Nicotiana langsdorffii* are well known as forming genetic tumors (SMITH, 1972). The system was used in screening for somatic hybrids between both species by CARLSON *et al.* (1972), and it seemed useful in our context too. Considering the genetics of tumor formation (SMITH, 1972), *N. glauca* was chosen as the DNA receptor. Pollen grains of *N. glauca* were germinated in DNA of different origins and used for pollination. The progenies obtained were tested for tumor formation by wounding. Compared with normal "dry" pollination there were statistically significant differences (0.1% level) in all treatments, *Petunia* and *N. langsdorffii* DNA being the most active. The results demonstrate clearly that nonspecific effects are involved, so one may ask whether any specificity could be detected. To test this, progeny were taken by selfing several plants from each experimental group and were wounded as above. Only *Petunia* DNA progenies, and *N. glauca* DNA progenies (level of significance 0.1%), showed a statistically significant reproducible stimulation in the number as

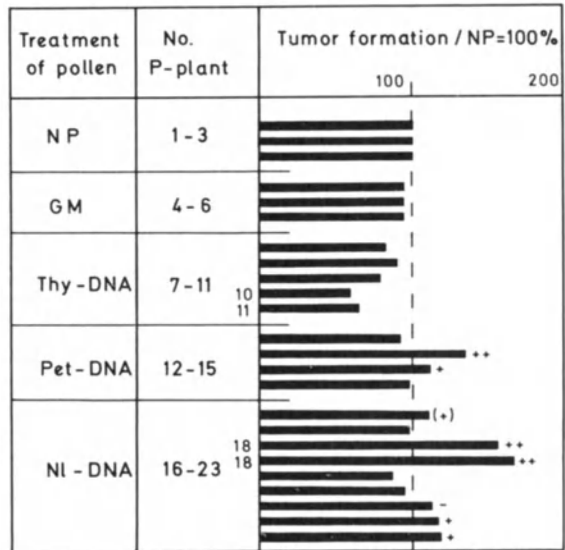

Fig. 10. Tumor formation in progenies from *Nicotiana glauca* parental plants (P-plants) derived from DNA-treated pollen. Treatment of pollen: *NP* = normal pollination, *GM* = germination medium, *Thy-DNA* = thymus DNA, *Pet-DNA* = *Petunia* DNA, *Nl-DNA* = *Nicotiana langsdorffii* DNA. In the diagram small (up to 5 mm diameter) and larger (more than 5 mm diameter) tumors were counted. If one counts only the larger tumors, there is no longer any difference between *Thy-DNA* and the *NP* or *GM* controls (HESS *et al.*, 1975)

well as size of the tumors (Fig. 10). If gene material for tumor formation should be transferred, then it shows once more a low expressivity because in most cases the tumors develop to a size of about 1 cm in diameter and then stop growing (HESS *et al.*, 1975). It should be remembered, however, that normally genetic tumors show a tendency to stop growth and form teratomic shoots.

6.3.2 Phages (and Plasmids)

As far as plasmids are concerned attempts have been made to transfer R-factors against kanamycin isolated from a kanamycin-resistant strain of *E. coli* by the pollen technique. Up till now effects have only been observed in the first generation and there is no real proof that they are genetical effects.

From parallel experiments using phages λpgal$^+$ and λplac$^+$, only the latter are presented (Fig. 11). (In the experiments with λpgal$^+$ comparable results were obtained.) Pollen of *Petunia* were incubated with phage λplac$^+$ and *E. coli* wild type DNA, and in the controls with phage λpgal$^-$ and *Petunia* DNA. The seeds obtained were put on 2% lactose nutrient agar. The seedlings in the λplac$^+$ and *E. coli* DNA series grew statistically significant better than in the best developing control series. Some fast growing seedlings (Fig. 12) in the λplac$^+$ series showed twice the specific β-galactosidase activity of the controls and of their weaker neighbors.

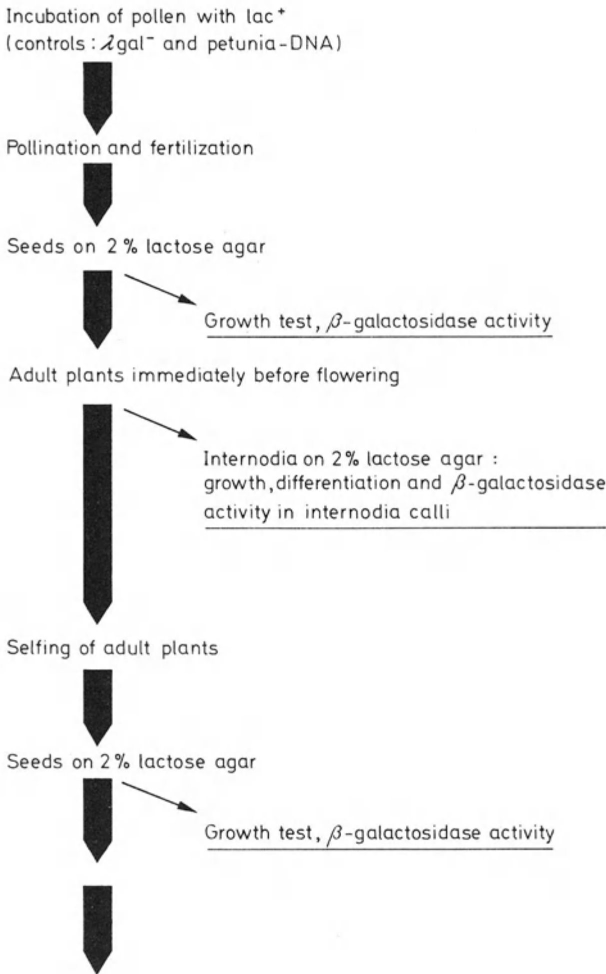

Fig. 11. Schedule of the λlac$^+$-pollen experiments

The λplac$^+$ experiments were further extended (the *E. coli* DNA series will follow) and the best growing seedlings in experiment and control were transplanted into soil. Immediately prior to flowering, internodes were taken off and put onto 2% lactose agar (and onto 2% sucrose agar for comparison). The development of callus, differentiation of shoots from the calli, and the β-galactosidase activity in these shoots were tested. In some cases all three parameters were higher in λplac$^+$ internodes than in λpgal$^-$ control internodes. The plants, from which these better-growing internodes had been taken, produced (by selfing) an offspring that grew significantly (0.1 level of significance) better than the best growing progenies of control plants (Fig. 13).

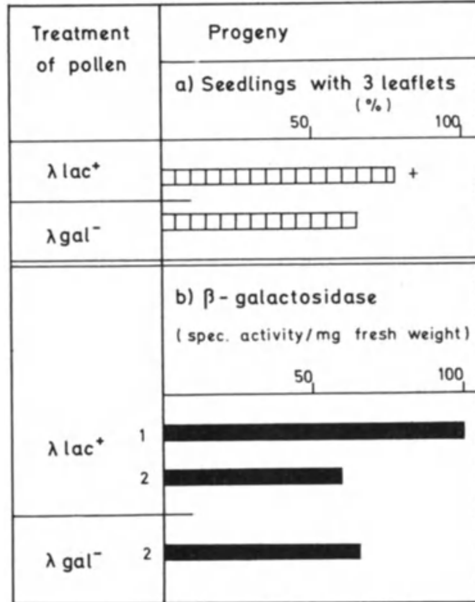

Fig. 12. Pollen as carriers for λplac$^+$, *1.* Generation. Pollen of petunia were treated with λplac$^+$ and in the control with λpgal$^-$. Seeds derived from fertilization using these pollen materials were put onto 2% lactose nutrient agar. Growth (number of seedlings with three visible leaflets 7 days after sowing; + = significance at the 1% level) and specific β-galactosidase activity were determined. λlac$^+$ *1* faster growing seedlings derived from λlac$^+$ treated pollen; λlac$^+$ *2* seedlings derived from λlac$^+$ treated pollen, with normal growth as in the λgal$^-$ controls (λgal$^-$2). (From HESS, 1976a)

Undoubtedly, a statistically significant genetic difference was introduced by treating the pollen with exogenous genetic material and maintained over at least two generations. As mentioned earlier, a statistical treatment seems absolutely necessary in all cases—and these so far outweigh the rest—where one expects quantitative differences. Furthermore, the *Nicotiana* experiments demonstrate clearly that one must not rely on the maintenance of a characteristic only over mitotic cell generations but also include a passage through meiosis. A full proof that we are dealing with a gene transfer would be the bacterial nature of the β-galactosidase at work in the phage experiments. Investigations to clarify this are in progress, but if we were not dealing with a gene transfer, the effects would be extremely difficult to explain.

7. Concluding Remarks

If we remember the four processes needed for a successful gene transfer, i.e. uptake, expression, integration, and replication, we have to state: in several systems *uptake* into the cytoplasm is proven (protoplasts) or is highly probable (cells in culture, organisms). *Expression* is mainly a matter of interpretation, at least for the moment, excluding the one case where the bacterial nature of an enzyme at work could be demonstrated (tomato cells + ∅ 80 plac$^+$). Matter of interpretation means that the data could hardly be explained any better than by an expression of exogenous gene material. But, as already mentioned, one should be aware that if not today, then tomorrow, other and perhaps better interpretations for the same

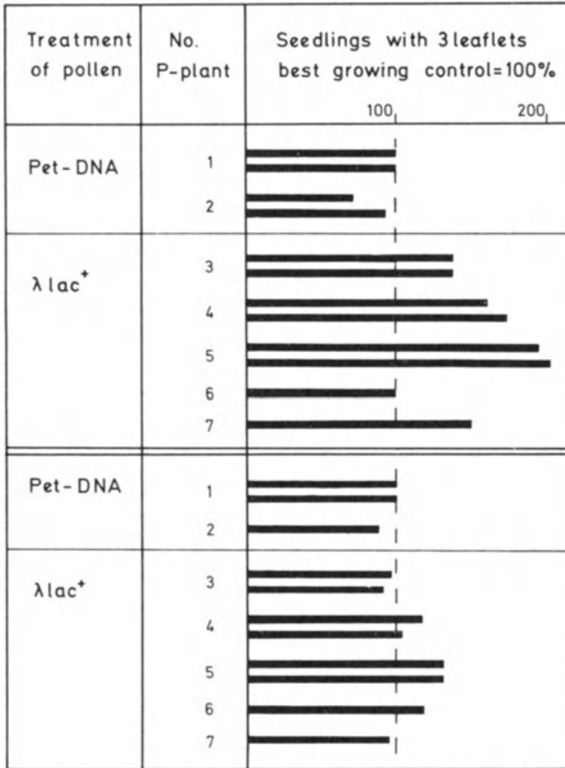

Fig. 13. Pollen as carriers for λplac$^+$, *2.* Generation. Pollen were treated with λplac$^+$ and in the control with *Petunia* DNA (Pet-DNA). Seeds derived from fertilizations using these pollen materials were put onto 2% lactose nutrient agar. The best-growing plants in experiment and control were transplanted into soil and selfed. Their offspring was tested on 2% lactose agar as indicated in Fig. 12. Two black bars behind the same number indicate repetitions. (From HESS, 1976a)

facts could be possible. Only a few data dealing directly with *integration* and *replication* are available, and these are not yet fully substantiated. All the other data about these two points are of an indirect nature, and once more a matter of interpretation in the sense just mentioned.

The question is whether the results obtained justify the efforts. First of all, it should not be forgotten that genetic engineering with higher organisms, especially with higher plants, is much more difficult than with bacteria and phages. Despite the difficulties, considerable benefit for theoretical genetics may result from these studies. For instance without transformation, transduction and transfection, our knowledge of bacterial genetics would be much poorer. Furthermore, in higher plant cells genetic markers could be introduced that would enable a screening after somatic hybridization, a method that seems most promising in combining closely related species (see Chap. IV.1). Most characteristics in higher organisms are determined by a whole set of genes. To transplant many genes scattered over the

genome one after the other will be at least very difficult, if not impossible. But there is a good chance of succeeding with single regulator genes and also with operon systems, especially if one combines the use of the restriction enzyme with phages, plasmids and perhaps the few known DNA plant viruses as carriers. Here the transfer of the nif^+ operon governing the fixation of the aerial N_2 would be of the highest importance.

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References see page 563.

4. Chloroplast Uptake and Genetic Complementation

K. L. Giles

1. Chloroplast Uptake

The genetic manipulation of plant and animal species has attracted a great deal of attention in the last few years. This has in part been due to the often frightening and bizarre forms to which, it has been suggested, this manipulation might give rise. None of these ideas has caught the popular imagination so strongly as that of "green men", photosynthetically active due to chloroplasts introduced into the skin. Such a suggestion at the present time is far beyond the bounds of possibility, though there have been reports of chloroplast uptake by both animal cells and plant protoplasts. These reports are reviewed below and their implications to the more theoretical aspects of the problem discussed.

1.1 The Uptake of Chloroplasts by Animal Cells

The occurrence of plant chloroplasts in animal cells was first demonstrated by Kawaguti and Yamasu (1965) in the marine gastropod *Elysia atroviridis*. The evidence that the structures observed were chloroplasts and not endosymbiotic algae was based on electron microscope examination and on comparison with the plastid structure in the siphonaceous alga *Codium fragile* on which the animals feed. No evidence was presented in this report to show that the chloroplasts were functional. The ultrastructure of chloroplasts in the digestive gland of *Elysia viridis* was studied by Taylor (1968), who showed that in this species too there was considerable similarity between the chloroplasts in the slug and those of *Codium tomentosum*, the alga upon which it feeds.

Yonge and Nicholas (1940) reported that the sacoglossan opisthobranch gastropod *Tridachia crispata* (Bergh) had symbiotic, unicellular zooxanthellae within the tissues of the gut. Upon re-examination by Trench (1969) these zooxanthellae were found to be absent, but the slugs did possess free chloroplasts, not whole algal cells, as a functional endosymbiont.

The chloroplasts were 2–3 microns in diameter and were in the cells lining the tubules of the digestive diverticulum. Electron microscopy revealed that the plastids were bound by a double membrane and possessed thylakoids. Osmiophilic granules, and what were interpreted as starch grains, were also seen in the chloroplasts. That these plastids were photosynthetically active was demonstrated by $^{14}CO_2$ pulsing of the slug in the light and dark, and autoradiography of histological preparations. Experimental animals in the light fixed appreciable amounts of ^{14}C, whilst dark control specimens fixed negligible amounts.

The life span of these plastids in the cell was estimated to be at least six weeks and possibly longer (TRENCH *et al.*, 1969). The chloroplasts appear to originate from siphonaceous algae and are not inherited but are acquired anew by each generation. That they are not passed on in the eggs as proplastids cannot be completely ruled out since electron microscope investigations have not been made.

Endosymbiotic chloroplasts have not been demonstrated in vertebrate tissues, but NASS (1969) showed that mouse fibroblasts in culture would incorporate isolated chloroplasts of spinach and African violet. These organelles were isolated and taken up by phagocytosis into the mouse cells, but despite this they were not contained in vacuoles or digestive vesicles. They could be seen segregating during mouse cell mitosis but were not seen to divide themselves, and were thus markedly diluted relative to the number of mouse L cells present within a period of 5–6 days. It was claimed that the plastids retained their structural integrity, though the electron microscope pictures included do not support this belief, the outer membrane having disappeared in all cases. Hill activity and the ability to fix carbon dioxide were reportedly maintained for 24 h.

Although very tight endosymbiosis appears to exist between some invertebrates and chloroplasts from the algae they eat, there is no suggestion that this relationship is dependent upon any form of genetic complementation. GILES and SARAFIS (1971, 1972) demonstrated that chloroplasts from *Caulerpa sedoides* (Brown in Turner) C. Agardh could be maintained in chicken egg whites for prolonged periods during which repeated chloroplast division occurred. Structural integrity was strictly maintained, and carbon fixation was demonstrated 96 h after isolation. These findings suggest that chloroplasts from at least some of the siphonaceous algae are autonomous for some of their metabolic functions, and this could partly account for their association with gastropod molluscs whose gut diverticulae must provide an environment suitable for the maintenance of these activities, to the ultimate advantage of the gastropod. The fact that these chloroplasts are not readily digested by the slug may be due to the rigescent integument (GILES and SARAFIS, 1974) which surrounds this type of plastid. It is interesting to note that when taken up by phagocytosis into mouse L cells, plastids of *C. sedoides* are maintained for several days and there is some suggestion that plastid division may occur within the animal cells. However, when the plastids are taken up by primary cultures of foetal lamb or bovine cells, the animal cells react by forming autosomal vesicles and die, releasing the chloroplasts into the medium. There does therefore seem to be some specificity of reaction to chloroplast uptake, at least in cultured mammalian cells.

The reverse experiment, introducing higher plant plastids into gastropod mollusc cells in vivo or in vitro, has not been attempted. It has proved difficult to date to find molluscs "uninfected" with chloroplasts in the wild. Maintaining animals in the dark for long periods does not rid them of plastids but frequently leads to death of the slug. Although gastropod mollusc larvae are free from chloroplasts as far as can be determined by optical microscopy, they die if they are not infected with plastids as they grow older. It would be of interest to determine the range of plastids capable of becoming the endosymbiont within these larvae.

1.2 The Uptake of Chloroplasts by Plant Cells and Protoplasts

Reports of the uptake of chloroplasts or other organelles by plant cells have necessarily been few. The rigid cell wall surrounding the plant cell is an effective barrier to all but the most minute particles, viruses normally entering the cell through plasmodesmata after damage to a neighbouring cell has allowed entry into the plant symplast. However, the slime moulds, members of the *Acrasiales*, have no such rigid cell walls and are capable of pinocytosing small particles. It is perhaps surprising that organisms such as the slime moulds have not been exploited more for this kind of study, since they provide a useful environment for the investigation of chloroplast survival in foreign plant cytoplasm. The ability of genetically dissimilar chloroplasts to survive in the same cell has been demonstrated in *Spirogyra* (VAN WISSELINGH, 1920), where in so-called mixed cells there exist chloroplasts both with and without pyrenoids. Such differences in chloroplast structure are maintained for many generations showing such plastids to be genetically different. In the iojap mutation of maize green and mutant white plastids are found in some cells (RHOADES, 1943). (see Sect. 3.3 for a fuller account.) In *Oenothera*, because of the biparental transmission of plastids during fertilization, STUBBE (1957) also could report mixed cells in the cotyledons and early leaves, though rarely in later leaves, of hybrid variegated plants. These findings add credence to the idea that genetically different chloroplasts may survive in the same cytoplasm.

An interesting communication from CARLSON (1973 a) reported the introduction of green tobacco chloroplasts into protoplasts from leaf mesophyll cells of a maternally inherited variegating albino mutant of *Nicotiana tabacum* (BURK and GROSSO, 1963). These protoplasts are albino and contain no functional chloroplasts. CARLSON (1973a) reported that they were capable of spontaneously pinocytosing normal green chloroplasts, and under suitable culture would regenerate whole plants, with a normal complement of green functional plastids. This, it was claimed, demonstrated that the chloroplasts were capable of utilizing information encoded by the protoplast nucleus. It is unfortunate that no methods or techniques used in this quite remarkable experiment were given in the publication, since to be acceptable as science it must be repeatable. The isolation of intact functional chloroplasts from tobacco is difficult, and no details of how this problem was overcome were given. The uptake of chloroplasts does not seem to occur spontaneously without the addition of some external compounds to the medium, but no details of methods used to induce plastid uptake were given. No information about the percentage of protoplasts taking up chloroplasts was given either.

POTRYKUS (1973) put forward some valid criticisms of Carlson's interpretation of this experiment. First, from experience with extrachromosomal, maternally inherited, stable, mutant plastids one should expect to get variegated plants when growing protoplasts via callus to a plant. One would expect from CARLSON'S (1973a, c) experiments to produce at least some variegated plants along with white and green ones. Secondly, since many extrachromosomally inherited, variegating, albino plants are periclinal chimeras of the "green/green/white" type (KIRK and TILNEY-BASSETT, 1967) the plastids of the epidermis are white because of their position but are genetically green. Such a cell would yield a protoplast

capable of regenerating a green plant without taking up any chloroplasts. Thirdly, the picutres presented by CARLSON (1973 c) show protoplasts that are apparently damaged and probably dead. The chloroplasts are ill-defined and seem to be adhering to the tonoplast or cytoplasm. This latter comment is concurred with by BONNETT and ERIKSSON (1974).

POTRYKUS and HOFFMANN (1973) succeeded in transplanting isolated nuclei of *Petunia hybrida* into protoplasts of *P. hybrida*, *Nicotiana glauca*, and *Zea mays*. About 0.5% of the protoplasts appeared to take up stained nuclei after treatment with 0.03% lysozyme in slightly hypotonic mannitol solution. That the nuclei were truly inside the protoplasts was demonstrated by following the position of the nucleus under the microscope while rolling the protoplast under the coverslip. Furthermore, when the protoplasts were burst under slight pressure it could be seen that the incorporated nucleus extruded together with the original organelles. This is a convincing demonstration of uptake and incorporation by the protoplast, but in these early days of experiment it is still important that uptake be proven without doubt. It is always difficult to be sure that particles, be they chloroplasts or nuclei, are truly within the plasmalemma when using only optical microscopy. The picture shown by POTRYKUS and HOFFMANN (1973), taken using oblique light and fluorescence, is not completely satisfactory. The ultimate demonstration of uptake of plastids must be by examination of serial thin sections by electron microscopy. Serial sections are necessary because particles could be taken in by a pinocytotic vesicle open to the exterior of the cell by a narrow neck, which might not be seen in a single section. Such a demonstration is still wanting.

POTRYKUS (1973) reported the transplantation of *P. hybrida* (cv. Cyatype) chloroplasts into the albino protoplasts from an extrachromosomally inherited, variegated *P. hybrida*. The chloroplasts were isolated by osmotic shock of isolated protoplasts, collected by centrifugation and resuspended in either 0.35 M mannitol or equiosmolar 0.21 sodium nitrate. The osmolarity at which the protoplasts burst, and hence the osmolarity to which the chloroplasts were first subjected, was not given. The chloroplasts were taken up by the protoplasts using either lysozyme or sodium nitrate treatment (see Sect. 2.3).

Isolation of chloroplasts by protoplast rupture, as reported by POTRYKUS (1973), is in our experience not likely to yield only single isolated plastids, but also groups of plastids. The groups tend to be held together, often with mitochondria, surrounded by membrane of either plasmalemma or tonoplast origin. This means that the sodium nitrate treatment, reported to be the more effective of the two methods tried, may well have had its effect by causing fusion of these sub-protoplasts with the albino protoplast. This conclusion is borne out by the pictures presented, showing the "uptake" of eight chloroplasts into a very localized area of the protoplast, a condition that indicates that fusion may have been involved. It is also mentioned that up to 20 plastids were taken in by some protoplasts, an unlikely event if they were taken in singly.

The most convincing report, and certainly the best pictorial record, of chloroplast uptake to date is that of BONNETT and ERIKSSON (1974). Using polyethyleneglycol treatment they succeeded in transferring chloroplasts of the alga *Vaucheria dichotoma* with high frequency into the protoplasts of *Daucus carota*. *Vaucheria*

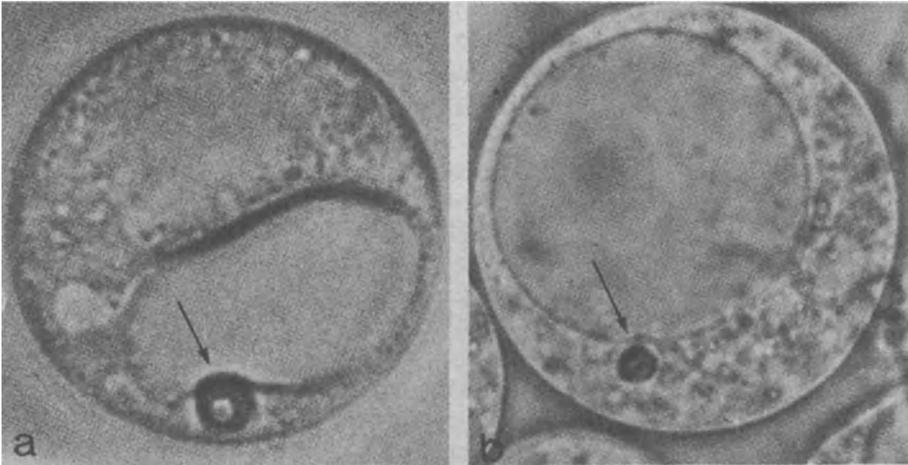

Fig. 1a and b. The induced uptake of chloroplasts of *Vaucheria dichotoma* (L.) Ag. by protoplasts of *Daucus carota* (L.) (a) A chloroplast *(arrowed)* in the cytoplasm of the protoplast causing a bulge into the vacuole; a clear indication that the plastid has been taken up. (b) Another chloroplast *(arrowed)* near to the vacuole and well within the protoplast cytoplasm. [(a) × 2500; (b) × 1600]. Courtesy of DR. HOWARD T. BONNETT from BONNETT and ERIKSSON, 1974

plastids were chosen for several reasons, the most important being their small size and their ability to withstand the necessary manipulations. It is significant that the chloroplasts of *V. dichotoma* possess a rigescent integument as described by GILES and SARAFIS (1974) and it is this structure that gives the plastid its structural stability at varying osmolarity. A great deal of trouble is taken to convince the reader that the chloroplasts are indeed taken into the cell cytoplasm; none was seen in the vacuole. Although further proof in the form of serial EM sections would be desirable, their evidence is convincing, especially their photograph (Fig. 1 a), kindly lent for reproduction here, showing a plastid in a cytoplasmic bulge into the vacuole. Figure 1 b shows another protoplast with a chloroplast near to the vacuole and well within the protoplast cytoplasm. Unfortunately, there is no report of any attempt to ascertain whether the chloroplasts were still active in any way before or after uptake.

The surprising difference between workers using animal cells and those using plant protoplasts is that the latter have ignored the obvious importance of the biological activity of the incorporated chloroplasts. Apart from mentioning that the plastids are functional, CARLSON (1973 a, c) gave no measurements of carbon fixation. POTRYKUS and HOFFMANN (1973) did not attempt a demonstration of activity of the nuclei. As will be discussed in a later section, it is extremely unlikely that any of the plastids used so far in uptake studies with higher plant protoplasts are functional. It is quite probable that it is the type of chloroplast eaten by gastropods, those symbiotic with marine siphonaceous algae, that offer the best hope of continued activity in both animal and foreign plant cytoplasm.

2. The Preparation of Material

2.1 The Preparation of Protoplasts

Three criteria must be upheld during the preparation of protoplasts for use in uptake experiments. The protoplasts must be (1) without any trace of cell wall, (2) highly cytoplasmic, and (3) alive. Traces of cell wall material attached to the protoplast interfere with uptake and can damage, and often rupture, intact isolated plastids. Relatively, cytoplasmic protoplasts seem more likely to take up particles than highly vacuolate ones, possibly because there is more cytoplasm to accomodate the added volume. Dead protoplasts are incapable of pinocytosis and as such incapable of meaningful uptake. Incorporation of particles into the cytoplasm of broken protoplasts is obviously not significant in the present context.

Any of the published methods, both in this volume and elsewhere, will give high yields of viable protoplasts, though slightly different osmotic conditions and enzymes might have to be used with different species. Highly cytoplasmic protoplasts can be found from meristematic tissues of both whole plant and callus origin. Both sources have their drawbacks however. Meristems from whole plants are time-consuming to dissect, and because of their size yield only a small number of protoplasts. The cells of the meristem are closely packed and thus slow the penetration of the cell wall-degrading enzymes, increasing the time necessary for exposure to enzymes. Rapidly growing meristematic callus gives a mixed population of protoplasts—some highly cytoplasmic, others highly vacuolate—the proportions of each depending on the precise growth conditions.

2.2 The Isolation of Chloroplasts

The methods used for the isolation of active chloroplasts from siphonaceous green algae and from higher plants differ only in detail, related to the different structures of the whole plant and plastids. The main consideration in both cases is the speed of isolation of the chloroplast from the cytoplasm and vacuolar contents. The latter is particularly important since in many plants heavy metal ions and phenolic compounds contained in the vacuole will cause almost immediate inhibition of the chloroplast enzymes.

GILES and SARAFIS (1971) described a method for the extraction and isolation of chloroplasts from the marine siphonaceous alga *C. sedoides*. In an attempt to simulate cell cytoplasm during the isolation, egg white was added to their medium, but subsequent experiments have shown that for the maintenance of activity the mineral salts medium described below is quite satisfactory.

Table 1. Isolation medium

$MgCl_2$	1 mM	EDTA	2 mM
$MnCl_2$	1 mM	Sorbitol	0.33 M
NaCl	10 mM	MES 50 mM	pH 6.5
$NaHCO_3$	20 mM		

The method currently used in this laboratory for the isolation of spinach chloroplasts is modified from BUCKE *et al.* (1966). Spinach is grown hydroponically in Hoagland's nutrient medium to minimize the build-up of heavy metal ions. Plants 2–3 weeks old are routinely used and only the youngest leaves chosen. These are immediately chilled in iced water and exposed for ten minutes to bright light 250 watts m^2. They are then quickly torn into thumb-nail sized pieces and put in a Virtis blender flask containing the solution detailed below. This solution is stored frozen and used in a half thawed state. Just before the leaf material is put in the blender 2 mM ascorbate is added to the mixture (Table 1).

As soon as the ascorbate and leaf material are added the whole is blended with the half-thawed medium for five seconds and immediately the slurry is filtered through three layers of Miracloth into a centrifuge tube free from all traces of detergent, which might lyse or break the chloroplasts, and centrifuged at $2000 \times g$ for 20 secs. The pellet is resuspended in the above medium and its activity measured with an oxygen electrode.

Activities of 50–80 μM oxygen/mg chlorophyll/h are routinely recorded with this method even at pH 6.5, a low pH for photosynthetic activity. Levels as high as 300–350 μM/mg/h can be recorded for spinach chloroplasts prepared in the same way but maintained at pH 8.2 (LYTTELTON, personal communication). This method works well for spinach, pea and *Chenopodium*, but does not yield intact active chloroplasts from all species. Treated with the same degree of loving care and attention tobacco chloroplasts are normally stripped of their outer membrane and are rapidly inactivated, although the stromal lamellae and grana hang together. There are no methods available that will give intact, active plastids of all plant species and, as yet, those species mentioned above are the only ones from which it can be said that good preparations can be made.

The preparation of chloroplasts from protoplasts has been suggested as a useful method of isolation. Most protoplasts after isolation are actively photosynthetic, though this activity decreases over a period of hours. Preparation of chloroplasts from protoplasts is suggested as being by simple lysis of the protoplast in hypotonic solution and subsequent release of the chloroplasts. In this laboratory such a method leads to the formation of groups of plastids, often accompanied by mitochondria, embedded in small pieces of cytoplasm, the whole being surrounded by a membrane, presumably of either tonoplast or plasmalemma in origin. When one speaks of uptake in this case one is really referring to fusion events, and these will be discussed as such later.

2.3 The Induction of Chloroplast Uptake into Protoplasts

Several methods for the induction of chloroplast uptake into plant protoplasts have been published, all of them depending either on some form of osmotic effect drastically plasmolysing the protoplast or on hypothesized changes in the charge pattern on the plasmalemma. CARLSON (1973 a, c) found it unnecessary to use any agent to promote chloroplast uptake but this is not the general finding. DAVEY

and COCKING (1972) when introducing bacteria into pea protoplasts stressed the importance of having the bacteria present during the plasmolysis of the cells. This does not seem to be necessary in the case of chloroplasts.

POTRYKUS (1973) describes two methods for the transplantation of chloroplasts of *Petunia*. In the first method protoplasts and chloroplasts were centrifuged in alternating layers together with 0.03% lysozyme, and then subjected to a hypotonic shock. The second method consisted in incubating the isolated single cells of *Petunia*, prepared by pectinase treatment, with 2% cellulase in 0.35 M mannitol, pH 5.4 at 20° C for 60 min. When the first protoplasts appeared, the single cells were transferred into a suspension of chloroplasts in 2% cellulase in 0.21 M sodium nitrate (equiosimolal with 0.35 M mannitol). The mixed suspension was incubated at 20° C for 15–30 min in a swing-out rotor at low speed at about $10 \times g$. The pellet was resuspended every 5 min. When all the cells had formed protoplasts the mixed suspension was washed clean of cellulase and free chloroplasts and the protoplasts examined. The possibility that the so-called uptake under these circumstances may actually be fusion as a result of the preparation of chloroplasts from the breakage of protoplasts in the presence of sodium nitrate has already been mentioned. The second of these two methods gave the best uptake but the condition of the chloroplasts after 15–30 min in 2% cellulase at 20° C is questionable. Isolated chloroplasts are normally maintained at, or near, 0° C to retain their activity, and an incubation of this length at 20° C is not calculated to maintain their integrity or metabolic activity, especially since cellulase preparations frequently contain appreciable amounts of protease.

BONNETT and ERIKSSON (1974) used a method involving plasmolysis by polyethylene glycol (PEG) to induce uptake of *Vaucheria* chloroplasts by carrot protoplasts. A dense mixture of protoplasts and chloroplasts was made up to a volume of 0.5 ml with protoplast culture medium (GLIMELIUS *et al.*, 1974). A volume of 1.5 ml of a solution containing 3 ml of protoplast culture medium and 7 ml of a 56% aqueous solution of PEG was added to the protoplast-chloroplast mixture. The final concentration of PEG was about 29%, a concentration known to promote cell aggregation and cell fusion without reducing cell viability (ERIKSSON *et al.*, 1974). After 10 min the mixture was diluted to 10 ml with a solution of 0.1 M $CaCl_2$ in 0.3 M sorbitol, and centrifuged for 3 min at $150 \times g$. The pellet was resuspended in 10 ml of the same solution, recentrifuged and resuspended in 2 ml of the protoplast culture medium.

As already mentioned (Sect. 1.2) this method seems to give good results, with up to 16% of the protoplasts taking up chloroplasts. KAO and MICHAYLUK (1974) observed some of the effects of PEG on protoplasts and mention a reduction in protoplast volume with a temporary wrinkling of the plasmalemma, aggregation of the protoplasts into groups, and eventual protoplast fusion. It is easy to see how any of these events might bring about chloroplast uptake.

New methods causing higher percentages of protoplasts to take in chloroplasts, and more importantly taking in larger numbers of chloroplasts must be sought. This last point is important because it may be that the number of plastids taken up may be critical in the further culture of the protoplast.

3. Genetic Complementation

Complementation between introduced organelles and the existing cell cytoplasm and organelles, can only occur if one partner lacks the ability to form a particular substrate or product, when the other partner is capable of either stimulating the formation of that substrate or product, or is capable of forming sufficient for both. Only the former alternative can be considered as genetic complementation, since the formation of new messenger-RNAs and the subsequent synthesis of new proteins must be considered as a prerequisite for true genetic complementation.

In order to demonstrate complementation in the case of chloroplast uptake by protoplasts it becomes necessary to be able to detect the synthesis of enzymes or new enzyme products, specifically coded for by the plastid, appearing in the cytoplasm, or the reverse process. To do this one must have some conception of what the chloroplast is capable of doing for itself.

3.1 How Autonomous is the Chloroplast?

The genetic and developmental autonomy of the chloroplast has for long been in dispute. In the light of evidence of the autonomy of plastids (RIDLEY and LEECH, 1970; GILES and SARAFIS, 1971, 1972) it seems probable that chloroplasts synthesize their own fatty acids. In the intact leaf most of the fatty acid synthesis takes place in chloroplasts (BROOKS and STUMPF, 1965). The cofactor requirements of synthesis, and the fact that malonate is incorporated, suggest that the pathway of fatty acid synthesis is similar to that worked out for animals (ALBERTS *et al.*, 1965).

Since protein is the major chloroplast constituent it is interesting to note that as early as 1959, TOLBERT showed that the products of photosynthesis formed from $^{14}CO_2$ by isolated chloroplasts included alanine and aspartate. Isolated spinach chloroplasts will incorporate radioactivity from ^{14}C-acetate into an amino acid fraction which includes glutamate (MUDD and MCMANUS, 1965). Chloroplasts seem therefore to be able to synthesize some of their amino acids. Synthesis of proteins in isolated chloroplasts of *Euglena gracilis* has been described (EISENSTADT and BRAWERMAN, 1963, 1964). It is of particular relevance to the present subject that incorporation of ^{14}C-leucine into protein took place rapidly during the first 15 min after isolation, but had virtually ceased after 30 min, indicating that the activity of isolated chloroplasts is short-lived.

The protein present in the largest quantity in the chloroplast is Fraction I protein. This name was first used by WILDMAN and BONNER (1947) to describe a high molecular weight soluble protein purified from spinach leaves. This protein can account for up to 50% of the total soluble protein in leaf extracts and is homogenous by electrophoretic and centrifugal criteria (KAWASHIMA and WILDMAN, 1970). It is located in the stroma of the chloroplasts, (LYTTLETON and TS'O, 1958) where it catalyses the CO_2-fixing step in photosynthesis (WEISSBACH *et al.*, 1956; PARK and PON, 1961).

The reported molecular weight of Fraction I protein purified from eucaryotic cells or blue green algae varies between 4.7×10^5 and 6.6×10^5 daltons (MCFADDEN, 1973). The most likely molecular weight is about 5.25×10^5 daltons. This

value is calculated from Fraction I crystalized from *Nicotiana* which could not be distinguished from Fraction I isolated from *Spinacia* (KAWASHIMA and WILDMAN, 1971). The intriguing point is that treatment of Fraction I protein with either urea (MOON and THOMPSON, 1969) or sodium dodecyl sulphate (RUTNER and LAVE, 1967) dissociates the molecule into large subunits (MW $5.2–6.0 \times 10^4$) and small subunits (MW $1.2–1.8 \times 10^4$). It seems from the work of RUTNER (1970), that the most likely composition of the undissociated Fraction I protein molecule is 8 large subunits with 8–10 small subunits.

Comparative studies carried out both between large and small subunits of Fraction I from a single species and between subunits from different species, have shown by amino acid compositions, (KAWASHIMA, 1969; MOON and THOMPSON, 1969; SUGIYAMA *et al.*, 1971) tryptic peptide analysis, (KAWASHIMA and WILDMAN, 1971; KAWASHIMA *et al.*, 1971) and immunological studies (KAWASHIMA *et al.*, 1971) that although the large subunit shares no similarities with the small subunit in the same species and small subunits from different species are highly dissimilar, the large subunits from different species are closely related. These findings may be of profound importance in studies concerned with chloroplast uptake into foreign cytoplasm.

Cycloheximide, an inhibitor of 80s ribosomal protein synthesis, has been used in studies on the synthesis of Fraction I protein in *Chlamydomonas reinhardi* (ARMSTRONG *et al.*, 1971; KAWASHIMA and WILDMAN, 1972) and higher plants (CRIDDLE *et al.*, 1970; ZUCKER, 1972) and the results have suggested the involvement of cytoplasmic ribosomes. CRIDDLE *et al.* (1970) have shown that the incorporation of labelled amino acids into the large subunit of Fraction I protein in greening barley leaves is preferentially inhibited by chloramphenicol, while that into the small subunit is inhibited preferentially by cycloheximide. These results indicate that the large subunit is synthesized by chloroplast ribosomes, and the small subunit by cytoplasmic ribosomes. These findings are supported by studies using isolated chloroplasts (BLAIR and ELLIS, 1972, 1973) and by KAWASHIMA'S (1970) finding that $^{14}CO_2$ is incorporated preferentially into the large subunit during short-term photosynthesis by *Nicotiana* leaves.

The nature of the mechanism regulating the synthesis of the two subunits in the different cellular compartments is still not clear. The large subunit synthesized in isolated chloroplasts does not interchange with that in preformed Fraction I protein, suggesting that the chloroplasts lack a pool of the small subunits (BLAIR and ELLIS, 1972, 1973).

Taken together, these findings point to a gloomy future for any chloroplast introduced into the cytoplasm of another species. Capable of forming only the larger subunit, and probably carrying no reserve pool of small units coded for by its parent nucleus, the plastid would be in an environment supplied only with foreign small subunits, dissimilar from its own. It would be able to function normally only if a successful hybrid Fraction I protein could be assembled from its large unit and the foreign small subunit. The smaller nuclear-coded subunit appears to have a regulatory function, since CO_2 fixation in its absence is very low and eventually ceases. It is perhaps relevant to the formation of Fraction I protein by chloroplasts and cytoplasm of hybrid origin that KAWASHIMA and WILDMAN (1972) showed, by intercrossing of several *Nicotiana* species, that the

genes for the small subunit of *N. tabacum* are dominant over those of *N. glauca* and probably *N. glutinosa*. Thus the problem of creating a hybrid Fraction I protein is fundamentally a genetic problem that is not solved by merely forming hybrid cytoplasm.

The central role of Fraction I in the functioning of the chloroplast presents a very real problem to the successful integration of the plastid in foreign cytoplasm. The reported long-lived activity of the chloroplasts of siphonaceous algae in animal cells presumably represents a long-lived form of the protein or possibly a form of it different from that found in higher plants. A ray of hope may be gleaned from the fact that the larger subunit seems very constant between species, suggesting that the production of hybrid forms of the enzyme might not be impossible.

3.2 Genetic Complementation by Chloroplast Uptake

To date there is no evidence of genetic complementation in the case of chloroplast uptake by either animal cells or plant protoplasts. The survival of the chloroplasts of the siphonaceous algae in the cells of various opisthobranch molluscs appears to be due to an inherent ability for survival outside plant cytoplasm, as is indicated by their longevity in egg white (GILES and SARAFIS, 1972). Egg white is only a reserve protein and the opportunity for genetic complementation is non-existant. It must be pointed out that in the case of uptake by cells no specific experiments have been reported which conclusively prove the absence of complementation, and until these are performed the possibility, however remote, that some facet of the plastid's existence in the cell is augmented by the nuclear activity of the host cell cannot be ruled out.

The number of reports of chloroplast uptake by plant protoplasts is fewer than that for animal cells, and here no specific studies have been carried out to assess complementation. As has already been pointed out (Sect. 1.2), biological activity has not been tested in the case of uptake by protoplasts, and much more information in this area is needed.

3.3 Genetic Complementation in Chloroplasts by Protoplast Fusion

Because of the easy identification of chlorophyll under the optical microscope, the chloroplast has been used in several demonstrations of genetic complementation by protoplast fusion.

The first demonstration of complementation was reported by GILES (1973, 1974) and involved the use of mutant lines of *Zea mays*. Fusion of protoplasts containing green chloroplasts from the striped mutant iojap, with protoplasts of the white deficient mutant containing only white undeveloped plastids, caused the greening of the white deficient chloroplasts over a period of 72 h. Fusion of white and green protoplasts from the iojap plants caused no such greening of the mutant iojap plastids.

In order to explain the type of genetic complementation occurring in the iojap-white deficient heterokaryons it is necessary to understand something of the genetics involved. Maize plants homozygous for the recessive gene iojap (ijij) exhibit a chlorophyll striping or variegation (JENKINS, 1924). The ij gene is situated on chromosome 7; its position in the linkage group has been determined with some precision through 3-point linkage tests. Cytological examination of the mesophyll cells of the white regions of ijij plants show that not only do the chloroplasts lack chlorophyll, but they are also much smaller than those of the normal green areas (RHOADES, 1943). The observation that F_1 plants of ijij constitution may be white or striped suggests that the modified plastids have a genetic continuity unaffected by the genetic constitution of the cell, and can thus be considered due to plastid mutation. This was shown to be correct when it was demonstrated that the aberrant plastids could remain and cause striping in IjIj plants (RHOADES, 1943). Thus it was shown that the iojap gene causes a high frequency of irreversible plastid mutations. The white deficient plants (wdwd) are due to a homozygous deletion of the terminal portion of a rearranged chromosome 9, caused by X-ray treatment (MCCLINTOCK, 1944). The deletion is caused by the "breakage-fusion-bridge cycle" (MCCLINTOCK, 1941) which occurs throughout the gametophyte mitoses, so that a resulting egg or pollen nucleus possesses a chromosome 9 with a freshly broken end, thus being deficient for minute terminal portions of the short arm of chromosome 9. After fertilization the cycle ceases to operate and the chromosome behaves normally. Plants homozygous for the chromosome 9 lacking the most terminal portions are pale yellow (pyd) rather than green, whereas those homozygous for a slightly larger deficiency lack both chlorophyll and carotenoids (wd).

The complementation that occurs in the iojap-white deficient heterokaryons is presumably due to the normal chromosome 9 donated by the iojap nucleus complementing the deficiency in the white deficient chromosome. Thus a lack of genetic information in one nucleus of the heterokaryon has been complemented by its presence in the other, allowing at least partial development of the chloroplasts. The failure of the green iojap to effect the formation of chlorophyll in the white iojap protoplast is because the condition is due to a plastid mutation, unaffected by the nucleus, and is not complemented by the presence of normal plastids. This latter point is shown by the existence in the plant of cells with mixed plastid populations. This explanation of the complementation is slightly complicated by the existence of the R. locus of the allele R^r. This allele controls, in conjunction with other factors, the pigmentation of both plant and seed (STADLER, 1946). It is possible that the greening that takes place in the white deficient protoplasts is at least in part due to interaction and complementation at the R. locus between the two genomes in the heterokaryon permitting greater pigmentation. However, this does not alter the fact that complementation of at least one, and possibly more, alleles occurs in these heterokaryons.

Because of the present impossibility of culturing maize protoplasts, no whole plants could be produced after complementation. Using mutants of tobacco, however, MELCHERS and LABIB (1974) have reported complementation between certain chlorophyll deficient and light sensitive varieties. They used the following

varieties sl_1 sl_2 (sublethal) (POVILAITIS and CAMERON, 1963), vi-A, (virescent) (SMITH, 1975), yg (yellow green) (NOLLA, 1934), and yc (yellow Crittenden) (VALLEAU, 1957). No fusion hybrids were found using yg and yc. Using sublethal and virescent, haploid plants were produced by anther culture, and the plants maintained in a low light intensity (800 lux), at 28° C with 80–90% humidity. After protoplast production and fusion, the protoplasts were plated and regenerated to whole plants as described by TAKEBE *et al.* (1971). For 48 h they were cultivated at approximately 300 lux, and after that at 3000 lux (ENZMANN-BECKER, 1973). After 2–3 weeks, cultures forming calli were picked out and grown at a light intensity of 8000–10000 lux. The hybrid plants expressed complementation for normal chlorophyll content and were not light-sensitive. That these plants were not simply revertants of the sublethal or virescent was demonstrated by the segregation of the progeny of the fusion hybrid into green, white and yellow seedlings.

This experiment is a clear indication of the usefulness of the genetic complementation of chloroplasts as a method for the selection of fusion products, a topic that will be discussed elsewhere in this volume. However, MELCHERS and LABIB (1974) does point out that in the case of two calli chosen for regeneration by their colour the plants were not dark green, showing that the method is not entirely effective.

Similar experiments using chlorophyll mutants of tobacco were described by SHVIDKAYA and GLEBA (1974). Here too it was shown to be possible to complement chlorophyll deficiency by protoplast fusion. While the protoplasts were still at the single heterokaryon stage no complementation was observed (BUTENKO, personal communication) and the reason for this is unclear. It is possible that the dedifferentation of the tobacco protoplasts prior to the formation of callus precludes the expression of chlorophyll synthesis, whereas in maize, where the protoplasts do not divide to form callus, dedifferentiation does not proceed to this point and chlorophyll synthesis may occur. However this may be, whole green plants could be regenerated after fusion of the protoplasts from mutant lines.

4. A Summary—The Agronomic Potentials of Chloroplast Uptake

From the foregoing sections it is abundantly clear that it is too early to assess the potential importance that chloroplast introduction might have for the improvement of agronomic species.

There are several reasons for this reluctance to evaluate the potential. Firstly, there are very few convincing reports of chloroplast uptake by protoplasts. In no case has biological activity been shown to exist in the chloroplasts, before or after uptake. In only one case has the protoplast as yet been shown to be capable of division after uptake (CARLSON, 1973), but the fact that chloroplasts were ever taken up in this experiment is open to some doubt.

Once active plastids have been introduced into foreign cytoplasm the question of genetic complementation arises. That isolated chloroplasts are capable of certain autonomous biological activity, when kept in ideal conditions at low temperatures over periods of a few hours at most, again raises the problem as to whether after introduction they would be capable of complementation or any activity. Prolonged periods at room temperature, or higher, are calculated only to reduce chloroplast activity perhaps to such a low level that genetic complementation becomes impossible. Nobody has suggested that apparently dead plastids, capable of no activity, can undergo a "miracle cure" in foreign cytoplasm and become active again. They may, however, serve as useful carriers of genetic information which by a process akin to transformation, (LEDOUX and HUART, 1968; HESS, 1969a, b) might be expressed in the host cell. There has been no demonstration as yet of any expression of genetic material carried by isolated chloroplasts after their uptake by protoplasts.

In the cases of complementation by protoplast fusion, chloroplasts are obviously capable of expressing the genetic information donated by their new environment. In fusion experiments however, they are transferred in their own cytoplasm to a hybrid form and are never as vulnerable to damage as the isolated plastid. At this stage of development it is very difficult to evaluate the potential of this type of corrective complementation, especially since its application is limited by the inability to culture the protoplasts of many species of both agronomic importance and otherwise.

An immediate use for chloroplast uptake studies is in the examination of the autonomy and genetics of the chloroplast. The hybrid nature of the Fraction I protein that must be built up in the cases of interspecific chloroplast uptake is an example of the kind of topic that can be studied now. Although not of direct agronomic application, this sort of study would certainly be of interest and quite possibly have far reaching implications for the whole field of somatic hybridization. It was pointed out a few years ago that one of the immediate applications of somatic hybridization might be as a genetic tool for the dissection of just this sort of problem (GILES, 1973). Although it would be as illadvised to say that chloroplast uptake has no agronomic potential as it would be to say that it has great potential, it can be said that such a potential has not yet been demonstrated.

5. The Potential for the Uptake of Blue-Green Algae

Since the earliest speculation concerning the origin of chloroplasts the blue-green algae, because their organisation is so similar to that of the chloroplast, have been put forward as the free-living ancestors of the chloroplast. They offer the advantage for uptake experiments that being free-living they are autonomous, indeed some of them, being capable of nitrogen-fixation, are even more autonomous than most higher plants. They also possess a rigid cell wall which makes them much more amenable to manipulation than a plastid. Encouragement for their future

use in uptake studies can be gained from the fact that there do exist natural symbioses within higher plants involving intracellular blue-green algae. *Gunnera* has intracellular blue-green algae actively fixing nitrogen, localised in mucilage glands at each node and at the growing tip. There is a tendency for the vegetative cells of the alga to change to heterocysts, a form lacking photosynthetic ability but seemingly having a higher concentration of the nitrogen fixing enzymes. The algae are enclosed in an interconnecting meshwork of membrane, possibly derived from the plasmalemma. The mode of uptake of these algae into the cells of *Gunnera* is still obscure, but the study of their uptake by protoplasts instead of the uptake of isolated chloroplasts might yield some interesting results!

References see pages 563.

5. Bacterial Uptake and Nitrogen Fixation

M. R. DAVEY

1. Introduction

A large number of micro-organisms from a diverse range of habitats are capable of fixing atmospheric nitrogen. Free-living fixers may be found in the soil, the sea, and in fresh water, some species being more abundant on the surface or in close proximity to plant organs, particularly in the rhizosphere and phyllosphere. Others form symbiotic associations within the confines of cells of certain plant organs, the *Rhizobium*-legume root nodule association being the most common. The nitrogen fixation process shows certain fundamental characteristics irrespective of the system in which it occurs. It involves the reduction of atmospheric nitrogen to ammonium in the presence of the enzyme nitrogenase, ammonium being the key intermediate in the synthesis of other nitrogenous compounds by living cells. The fixation of atmospheric nitrogen by free-living and symbiotic micro-organisms is of the utmost importance to world agriculture, since it is the principal process for maintaining the nitrogen fertility of soils. However, in spite of the diversity of the biological systems in which the process takes place, there remains a world shortage of dietary protein related to the inadequate reduction of nitrogen to ammonium. Legume species are able to thrive even on nitrogen-deficient soils, and their ability to improve soil fertility and their use in human consumption and animal feedstuffs has been appreciated by ancient and modern civilisations. Non-leguminous crop plants require a supply of organic or inorganic nitrate fertilisers as a source of nitrogen, which imposes a heavy economic burden on the less affluent countries of the world. The shortage of protein and the cost of fertilisers have stimulated interest and research to gain more understanding of the physiology and genetical control of nitrogen fixation in both symbiotic and free-living micro-organisms (see reviews by POSTGATE, 1971; STREICHER *et al.*, 1972; QUISPEL, 1974).

2. Tissue Culture Systems for Studying Nitrogen Fixation

Experimental investigations of nitrogen fixation in legumes is hampered by the complexity of the whole plant system. Plant tissue culture techniques have provided an opportunity to study some aspects of the higher plant-bacterial symbiosis under in vitro conditions. Early work in this field utilised cultures of excised legume roots (RAGGIO *et al.*, 1957, 1959), and although root nodules developed in response to infection by *Rhizobium*, this experimental system still suffered from many of the complexities of the intact plant. In an attempt to simplify the experimental arrangement, VELICKY and LARUE (1967) used soybean root cell cultures. Although stimulation of cell differentiation and lignification apparently resulted in the presence of *Rhizobium*, no evidence for intercellular

bacterial symbiosis was found. The experiments of HOLSTEN *et al.* (1971) were more successful in establishing a symbiotic nitrogen-fixing relationship between plant cells and micro-organisms in vitro. These workers used cell suspension cultures initiated from explants excised from primary roots of soybean (*Glycine max* v. Acme) shortly after seed germination. Addition of *Rhizobium japonicum* to the cultures was followed by the development of infection threads within the cell aggregates, and the subsequent invasion of 1 to 10% of the callus cells by the bacteria. Ultrastructural studies showed infection in the in vitro system to be similar to the process as it occurs in cells of developing soybean root nodules (GOODCHILD and BERGERSEN, 1966). The bacteria were released from the infection threads into membrane-bounded vesicles in the cytoplasm of the callus cells, the membranes of the vesicles developing from the plasmalemma of the host cells. The micro-organisms continued to divide until they filled the cytolpasm of the host cells, after which they ceased division and developed electron-transparent regions in their own cytoplasm. These regions are thought to be accumulations of a polymer of β-hydroxybutyrate, which in root nodules is considered to be a characteristic inclusion of bacteroids, the form of *Rhizobium* cells effective in nitrogen fixation. The callus symbiosis displayed nitrogen-fixing activity as measured by the reduction of acetylene to ethylene using gas chromatography, amounting to about 1% of the fixation rate of intact nodules. It was markedly dependent upon the supply of exogenous hormones provided in the culture medium. Studies by other workers have confirmed the development of nitrogen fixation in soybean callus cultures following infection by *Rhizobium* (CHILD and LARUE, 1974), but have failed to demonstrate conclusively the penetration of bacteria into the cytoplasm of the host cells followed by change of the micro-organisms to the bacteroid form. In addition, the synthesis of leghaemoglobin, the pigment present in functional nodules which is considered to be an essential component of the oxygen transport system between the membranes of the host-derived vesicles enclosing the bacteroids and the bacteroids themselves, has not been confirmed in the in vitro cell systems. Such results have led to the suggestion that the presence of bacteria in the intercellular spaces, in close association with the cells of cultured tissues, may be sufficient for a nitrogen-fixing symbiosis to be established. Recent experiments with cultured cells have indicated that some slow-growing strains of *Rhizobium* are able to fix nitrogen in association with cultures isolated from non-leguminous species of higher plants (CHILD, 1975; SCOWCROFT and GIBSON, 1975). This data suggests the genetic information for nitrogenase is encoded in *Rhizobium*, and that its expression depends on a diffusable factor secreted by higher plant cells.

3. Isolated Protoplasts: a Single Cell System for Nitrogen Fixation Studies

Recent advances in the enzymatic isolation of higher plant protoplasts have enabled these naked cells to be prepared from most plant tissues and from many lines of cultured cells. Several protoplast systems will undergo wall regeneration

in culture followed by cell division to form callus masses, and whole plants can be recovered from these callus cultures in a few species (see reviews by COCKING, 1972; BAJAJ, 1974a). A population of isolated protoplasts provides a unique single cell system for attempting to modify higher plant cells to perform specific functions (COCKING, 1973), and for investigating microbial-higher plant associations. An important modification would be genetic alteration to confer the ability of nitrogen fixation on cells of non-leguminous plants. There exist three experimental approaches for investigating the possibility of modifying higher plant cells in this way. Modification may perhaps be achieved by uptake into protoplasts of *Rhizobium* or bacteria able to fix nitrogen in the free-living state (eg. *Azotobacter*), by transfer of genetic material controlling the nitrogen fixing association with *Rhizobium* from legumes to non-legumes by somatic hybridisation of isolated protoplasts, or by uptake and expression of genetic material controlling nitrogen fixation in free-living micro-organisms (the nitrogen fixation or "*nif*" genes).

4. Uptake into Isolated Plant Protoplasts: Activity at the Plasmalemma

Attempts to obtain uptake of intact bacterial cells into protoplasts stemmed from earlier observations on the uptake of particulate materials into isolated higher plant protoplasts. The plasmalemma of an isolated protoplast should not be thought of as a tightly stretched surface, but as a membrane capable of altering its shape by developing outgrowths as well as invaginations. Extensive ultrastructural studies have demonstrated the frequent occurrence of vesicles within the cytoplasm of isolated protoplasts. Each vesicle is surrounded by a single membrane which in many cases originates as an invagination of the plasmalemma into the cytoplasm. The open end of the invagination then seals as a result of membrane fusion to release the vesicle into the cytoplasm. Vesicles vary considerably in size and are thought to be of two types, namely plasmolytic and endocytotic. Plasmolytic vesicles are often several μm across and originate as gross infoldings of the plasmalemma into the cytoplasm as the protoplast contracts during plasmolysis. Endocytotic vesicles are smaller and arise both during wall removal and following protoplast isolation. The extent to which endocytosis normally occurs in intact plant cells is uncertain, and direct evidence for this phenomenon in plants has come mainly from studies with isolated protoplasts. Removal of the cell wall appears to stimulate activity of the surface membrane, the extent of endocytosis depending on the protoplast system and the constituents of the plasmolyticum used to stabilise the naked cells. As endocytosis generally involves an initial adsorption onto the plasmalemma of the material to be taken up, then the surface charge of this material in relation to the charge of the plasmalemma is critical. Polycations such as poly-L-ornithine can alter the charge on the surface membrane of the protoplasts and may be used at low concentrations (1 to 2 μg/ml) to stimulate uptake.

In order to demonstrate uptake by endocytosis, the freshly isolated protoplasts are incubated in a suitable plasmolyticum containing the material to be

taken up for a period of a few minutes to several hours. A range of particulate materials including viruses (eg. tobacco mosaic virus), organic macromolecules (eg. ferritin), and inert particles (eg. polystyrene latex spheres and colloidal thorium dioxide) have been observed by electron microscopy to be taken up into membrane-bounded vesicles in the cytoplasm of protoplasts isolated from locule tissue from immature fruits of tomato (*Lycopersicon esculentum* v. Ailsa Craig), and leaf mesophyll tissue of tobacco (*Nicotiana tabacum* cv. Xanthi) (COCKING, 1965, 1966, 1970; DELEO and COCKING, 1967; COCKING and POJNAR, 1969; POWER and COCKING, 1970; WILLISON *et al.*, 1971; WITHERS and COCKING, 1972). Latex spheres up to 0.25 μm in diameter are the largest particles observed to enter protoplasts by endocytosis (MAYO and COCKING, 1969).

5. Uptake of Bacteria into Plant Protoplasts

Ultrathin sections of isolated protoplasts suggested that particles considerably larger than 0.25 μm could be accommodated within some of the cytoplasmic vesicles of isolated protoplasts. Indeed, some vesicles were considered large enough to contain whole micro-organisms. This hypothesis was investigated by studying the uptake of bacterial cells (*Rhizobium leguminosarum;* Rothamsted Culture Collection Cat. No. 1007) into protoplasts of pea (*Pisum sativum* cv. Little Marvel) leaf mesophyll cells. The reasons for this choice of experimental material were two-fold. First, it was considered that since this bacterium normally induces nodules effective in nitrogen fixation on roots of the host plant, this micro-organism would be the most compatible bacterium to use with cells from other parts of the same legume species. Second, in the event of successful uptake, the behaviour of bacteria in the cytoplasm of the protoplasts could be studied during subsequent culture of the naked cells.

Experiments were performed to determine whether *Rhizobium* could be taken up into isolated pea leaf protoplasts by endocytosis. The *Rhizobium* cells were grown in shake cultures in liquid mannitol yeast-water medium, and exponentially growing cells harvested for use three days after inoculation. After washing to remove the culture medium, the bacterial cells were suspended at a density of approximately 2.0×10^8/ml in a plasmolyticum (25% w/v sucrose solution) containing freshly isolated mesophyll protoplasts (5.0×10^5/ml). The protoplasts were prepared from fully expanded leaflets of 5–7 week old pea plants using a mixture of 5% w/v meicelase (Meiji Seika Kaisha, Ltd., Tokyo) with 5% w/v macerozyme (All Japan Biochemical Co. Ltd., Nishinomiya) in 25% w/v sucrose (pH 5.8) for 20 h at 25 C. in the dark (DAVEY and SHORT, 1973). The protoplasts were incubated with *Rhizobium* for periods of up to 12 h, but uptake of bacteria did not occur even when the naked cells were treated with polycationic stimulators of endocytosis. This experiment confirmed that endocytotic uptake into protoplasts is restricted to small particles, as suggested by the earlier experiments of other workers.

The method which resulted in the successful uptake of *Rhizobium* into pea leaf mesophyll protoplasts involved entry of the bacteria into plasmolytic vesicles. In

the method described (DAVEY and COCKING, 1972), leaflets excised from young pea plants were wetted by immersion in 1% v/v sterile "Teepol" (BDH Chemicals Ltd.) for 3 min, and surface sterilised in 1.5% v/v sodium hypochlorite solution containing 10–14% available chlorine (10 min). The sterilant was removed by eight successive washes with sterile water. The lower epidermis was removed by peeling with fine forceps, and the leaf pieces were incubated with their exposed mesophyll in contact with a filter-sterilised enzyme mixture of cellulase and pectinase. 1.5 g fresh weight of peeled tissue was floated on the surface of 5 ml of enzyme contained in a 5 cm petri dish. The enzyme mixture and incubation conditions were the same as those previously described for the isolation of pea mesophyll protoplasts except that the enzyme contained *R. leguminosarum* at a density of approximately 2.0×10^8/ml. The bacteria were harvested from exponentially growing cultures immediately before use. Protoplasts released during the enzyme incubation were freed of leaf debris by straining through a fine wire gauze, and collected by centrifuging the protoplast-enzyme-bacterium suspension (225 g; 5 min). The film of intact, floating protoplasts was washed to remove enzymes and excess bacteria from the surface of the protoplasts by re-suspending 6 times in fresh plasmolyticum followed by centrifugation (225 g; 5 min). Protoplasts were fixed, embedded, and sectioned for electron microscopy by a routine procedure (DAVEY and COCKING, 1972, DAVEY and SHORT, 1972).

6. Fine Structural Studies of Bacterial Uptake

Ultrastructural observations showed the presence of *Rhizobium* localised within membrane-bounded vesicles in the cytoplasm of some of the isolated pea mesophyll protoplasts. Many of the vesicles containing bacteria were deep within the cytoplasm, frequently internal to the other organelles such as chloroplasts and mitochondria, and also within transvacuolar cytoplasmic strands (Fig. 1A–C). This location of the membrane-bounded vesicles suggested they were completely closed to the exterior of the protoplasts, with little possibility of them being merely invaginations into the cytoplasm. Similar results have also been obtained using another legume mesophyll protoplast system. Mesophyll protoplasts were isolated from expanded simple leaves of 8-day-old cowpea (*Vigna sinensis* cv. Blackeye) seedlings in the presence of *R. japonicum* (Rothamsted Culture Collection Cat. No. 3824), under incubation conditions similar to those described for the isolation of pea mesophyll protoplasts. Bacterial cells were again found localised within membrane-bounded vesicles in the cytoplasm of the isolated cowpea mesophyll protoplasts (Fig. 1D, E).

It is difficult to access accurately the number of bacteria taken up into a single protoplast from thin sections examined in the electron microscope. Analysis of several experiments have shown that approximately 5% of both pea and cowpea protoplasts contained *Rhizobium* cells, some with as many as 10 bacteria per protoplast section. Two or more bacteria were present in some vesicles. The walls of the bacteria are resistant to degradation by the hydrolytic enzymes used to digest the walls of the higher plant cells. Morphologically the bacteria appeared

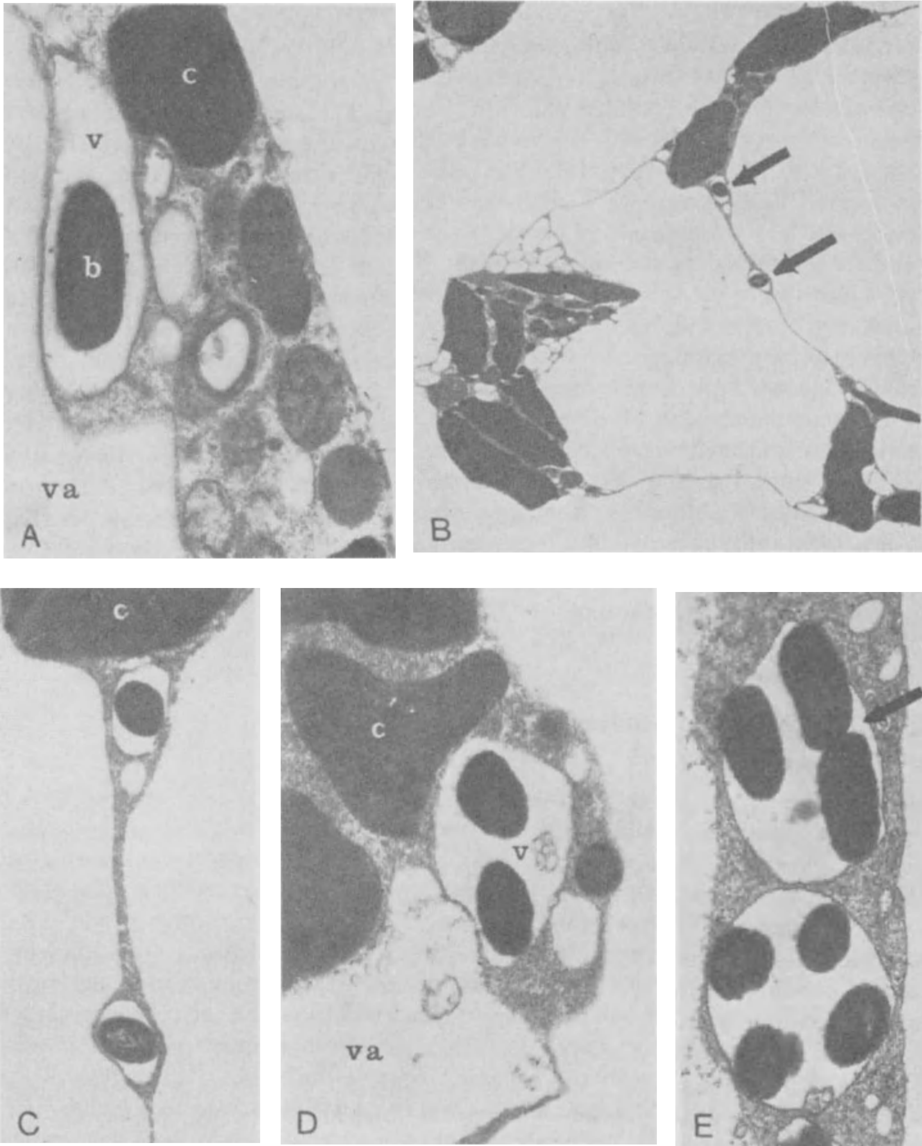

Fig. 1A–E. Uptake of *Rhizobium* into leaf protoplasts. (A) A bacterium (*R. leguminosarum*) in a membrane-bounded vesicle in the cytoplasm of an isolated pea mesophyll protoplast. *b* = bacterium; *c* = chloroplast; *v* = vesicle; *va* = vacuole. × 19500. (B) Bacteria *(arrowed)* in a transvacuolar cytoplasmic strand of a pea protoplast. × 4500. (C) The cytoplasmic strand of *b* showing bacteria within membrane-bounded vesicles. *c* = chloroplast. × 12500. (D), (E) Bacteria (*R. japonicum*) in membrane-bounded vesicles in the cytoplasm of isolated cowpea mesophyll protoplasts. One bacterium is constricted *(arrowed)*. *c* = chloroplast; *v* = vesicle; *va* = vacuole. × 15250

unaffected by the plasmolyticum used to stabilise the higher plant protoplasts, or by being enclosed within vesicles in the cytoplasm of the leaf mesophyll protoplasts.

Uptake of bacteria into mesophyll protoplasts only occurred during enzymatic digestion of the walls of the higher plant cells. The uptake process is therefore thought to occur by engulfment of the bacterial cells into vesicles formed by invagination of the plasmalemma during plasmolysis and concomitant degradation of the cell wall, rather than by a strictly endocytotic process. As the bacteria are included in the enzyme mixture, they are able to penetrate to the plasmalemma immediately the higher plant cell wall is weakened by the hydrolytic enzymes. It must be emphasised that protoplast contraction is not an immediate all-or-nothing reaction when cells are placed in a plasmolysing solution. There may occur considerable activity of the surface membrane over a period of perhaps several hours. Bacteria arriving at the surface of a protoplast while the plasmalemma is still infolding may be taken up into invaginations formed by the surface membrane and eventually come to lie in the membrane-bounded vesicles which are released into the cytoplasm. The uptake of *Rhizobium* is probably assisted by its motility. Uptake may be governed to some extent by the structural characteristics of the protoplasts, and the amount of space available in the cytoplasm into which vesicle formation can occur. Thus, closely packed, peripherally arranged chloroplasts may limit uptake into some mesophyll protoplasts, and leaf epidermal protoplasts with less plastids (DAVEY *et al.*, 1974) may be more suitable for studies of this kind.

7. Comparison of Rhizobium in Leaf Protoplasts and Infected Cells of Root Nodules

Since the membranes of the vesicles containing the bacteria are derived from the plasmalemma of the host protoplast, their origin is directly analogous to that of the membrane enclosing *Rhizobium* which enter root nodule cells via the normal infection thread (GOODCHILD and BERGERSEN, 1966). The morphological changes that the *Rhizobium* cells undergo in the whole plant system as they change to the bacteroid form active in nitrogen fixation have already been discussed. Division of the bacteria ceases as the cells undergo this change. At present nothing is known of the fate of *Rhizobium* taken up into legume leaf protoplasts; whether the bacteria will undergo similar changes to those described in cells of root nodules remains to be seen. Constricted bacteria have been observed in some vesicles (Fig. 1E), but it is not clear whether they began to divide within the vesicles following uptake, or whether they had already commenced division prior to uptake. Experimental evidence suggests, in fact, that the bacteria do continue to divide within the protoplasts when the naked cells are maintained in liquid or agar culture medium. This presents certain technical problems, since during the early stages of culture the host protoplasts are still naked or have only a thin regenerated cell wall. In the absence of a wall of normal thickness, there is little to

restrain the pressure outwards exerted by the dividing bacteria, which results in bursting of the host protoplasts. This difficulty may perhaps be overcome by using auxotrophic strains of *Rhizobium*, whose division within the host protoplasts may be suppressed by the omission of a specific metabolite from the culture medium until wall regeneration by the protoplasts has reached an advanced stage. Although pea leaf mesophyll protoplasts will undergo wall regeneration followed by cell division (CONSTABEL *et al.*, 1973), there are other legume protoplast systems available with a faster growth rate and a higher plating efficiency. Those isolated from soybean callus (*Glycine max;* KAO *et al.*, 1970), leaf mesophyll cells of bean (*Phaseolus vulgaris* cv. Pinto; PELCHER *et al.*, 1974) and cowpea (*Vigna sinensis* cv. Blackeye; DAVEY *et al.*, 1974 a), readily undergo wall regeneration and division in culture. Protoplasts from these sources may be more suitable as experimental systems for studying the behaviour of *Rhizobium* within the cytoplasm of legume cells under in vitro conditions, and to monitor the development of any symbiotic nitrogen-fixing association which possibly may develop.

8. Application of Rhizobium Uptake into Legume Protoplasts to Other Systems

Isolated pea and cowpea leaf mesophyll protoplasts provide model systems for investigating the uptake of *Rhizobium* into cells of the plant body where these bacteria do not normally penetrate under natural conditions. Entry of bacteria into protoplasts by the plasmolytic method described should not be restricted to the uptake of *Rhizobium* into legume protoplasts, but this technique should be applicable to other micro-organisms and protoplast systems, including protoplasts isolated from non-legumes. The demonstration of nitrogen fixation by *Rhizobium* in the presence of cultured cells from non-leguminous species (CHILD, 1975; SCOWCROFT and GIBSON, 1975) emphasises the need to investigate the uptake of *Rhizobium* into protoplasts of non-legumes, particularly those of agriculturally important crops and forage plants, and to study the subsequent behaviour of the bacteria within the higher plant cells. Certain technical difficulties may be experienced in applying the uptake technique to some micro-organisms. If they are non-motile or larger than *Rhizobium* (rods, 0.5–0.9 × 1.2–3.0 μm) then it is difficult to maintain intimate contact between the micro-organisms and the higher plant tissue during the enzyme incubation period required to digest the walls of the higher plant cells. There have been reports of the uptake of large particles such as isolated nuclei and chloroplasts into protoplasts using lysozyme (POTRYKUS, 1973; POTRYKUS and HOFFMANN, 1973) and polyethylene glycol (BONNETT and ERIKSSON, 1974; ERIKSSON *et al.*, 1974). These treatments may provide alternative methods of increasing the frequency of uptake of micro-organisms into higher plant protoplasts.

At present, there are no reports describing the uptake of micro-organisms other than *Rhizobium* into higher plant protoplasts. The uptake of blue-green algae and bacteria, particularly those which fix nitrogen in the free-

living state such as *Azotobacter*, may produce new endosymbiotic relationships with higher plant cells. Uptake of free-living nitrogen fixers could confer the desired modification of nitrogen fixation on the higher plant cells in one of two ways. The bacterial cells could become established and continue to divide and fix nitrogen within the host cells. Alternatively, it may not be necessary for the bacteria to remain intact within the host cytoplasm. They could undergo intracellular degradation with release of their DNA. It is conceivable that this genetic material, particularly the genes controlling nitrogen fixation, could become incorporated into the genome of the higher plant cell. DILWORTH and PARKER (1969) have postulated that the development of a *Rhizobium*-legume symbiosis may have originated in this way during evolution, part of the bacterial DNA concerned with the production of nitrogenase being transferred to the legume to become part of the higher plant DNA complement. As a direct result of this transfer *Rhizobium* and the legume became dependent on their symbiotic association for the production of nitrogenase.

9. The Use of Isolated Protoplasts Containing Bacteria in Somatic Hybridisation Studies

Mention has already been made of the alternative experimental approaches that can be adopted in an attempt to modify non-leguminous higher plant cells to fix nitrogen. One of these involves somatic hybridisation by fusion of isolated protoplasts. Since the first demonstration that isolated higher plant protoplasts could be induced to undergo intra- and inter-specific fusion when treated with sodium nitrate solution (POWER *et al.*, 1970), several workers have attempted to improve the technique to obtain a high percentage of fusion while still retaining a high level of protoplast viability. The most successful method appears to be treatment of protoplasts with polyethylene glycol (ERIKSSON *et al.*, 1974; KAO and MICHAYLUK, 1974; WALLIN *et al.*, 1974). This technique has been employed to fuse protoplasts from a number of plants, including those of legumes with non-legumes (KAO *et al.*, 1974). Tetraploid cells or cells which are multiples of this ploidy level in the root cortex of leguminous plants normally become infected during root nodule development. Assuming that protoplasts isolated from cells of other tissues such as the leaf mesophyll of legumes also carry the genetic information necessary for the *Rhizobium*-legume association to be established, then the fusion process may convey this genetic information to the non-legume. Callus regeneration followed by whole plant recovery from the heterokaryons may produce a hybrid which retains the morphological characteristics of its non-legume parent, but which shows the legume characteristic of being able to associate symbiotically with *Rhizobium*.

In somatic hybridisation experiments it may be advantageous to fuse legume protoplasts, particularly those which have taken up *Rhizobium* into vesicles in their cytoplasm, with protoplasts isolated from non-legumes. In addition, protoplasts which naturally contain a large number of *Rhizobium* bacteroids within

membrane-bounded vesicles in their cytoplasm can be isolated enzymatically from cells of the infected tissue of young root nodules of soybean (*G. max* cv. Grant (DAVEY *et al.*, 1973). These protoplasts are released when tissue slices (about 0.3 mm thick) of young nodules excised from 16–24 week-old plants are incubated in a mixture of 1.5% w/v cellulase R 10, 0.5% w/v macerozyme R 10 (Kiniki Yakult Ltd.) with 1.0% rhozyme HP150 (Rohm and Hass Co. Ltd., Philadelphia) in 12% w/v mannitol (pH 5.8) for 5 h at 20° C in the dark with gentle agitation on a horizontal shaker. The material is then teased apart and incubated without shaking for a further 1 h. The protoplast-enzyme mixture is strained through a fine wire gauze to remove debris. Protoplasts are allowed to settle, and washed by changing the supernatant several times with fresh plasmolyticum. Unlike protoplasts isolated from other plant tissues, root nodule protoplasts generally remain elongate in shape (Fig. 2A). Their failure to become spherical following removal of the cell wall probably results from the large number of bacteroids within vesicles in their cytoplasm (Fig. 2B, C), and the absence of a large central vacuole. Isolated root nodule protoplasts may prove useful in somatic hybridisation experiments aimed at establishing *Rhizobium* in the cytoplasm of non-leguminous cells. Furthermore, they provide a system with which to make a direct structural comparison of protoplasts from other plant tissues which have taken up micro-organisms under experimental conditions.

10. Transfer of Genes Controlling Nitrogen Fixation

The precise genetic control of nitrogen fixation in legumes is still unknown (DILWORTH and PARKER, 1969). Plant genes are involved (NUTMAN, 1969), since the protein moiety (globin) of leghaemoglobin is coded for by the plant. However, it is still uncertain whether nitrogenase genes of root nodules originate from plants or the infecting bacteria, although localisation of most of the nitrogenase within the bacteroids favours a bacterial origin (BERGERSEN, 1971). The complexity of the legume symbiosis and the inability of *Rhizobium* to fix nitrogen in the free-living state has prompted geneticists to use free-living nitrogen fixing bacteria to investigate the genetic control of the fixation process. *Klebsiella pneumoniae* has featured as an experimental organism in much of the recent work (STREICHER *et al.*, 1972). This bacterium is closely related to *Escherichia coli* with a similar genetic map, the structural and regulatory genes controlling nitrogenase in *Klebsiella* (the "*nif*" genes) being found near to those controlling histidine synthesis ("*his*" genes). Under natural conditions *E. coli* does not fix nitrogen, but transfer of the *his*-linked *nif* region from *Klebsiella* to *E. coli* by conjugation under experimental conditions has resulted in the expression of nitrogen fixation in the recipient *E. coli* strain (DIXON and POSTGATE, 1971, 1972; CANNON *et al.*, 1974). This demonstration of gene transfer in bacteria has stimulated many workers to consider the possibility of transferring the "*nif*" genes from micro-organisms to higher plants. Isolated protoplasts would feature as an important experimental system with which to carry out such investigations, since their ability to take up DNA has already been demonstrated (OHYAMA *et al.*, 1972; HEYN and SCHILPEROOT,

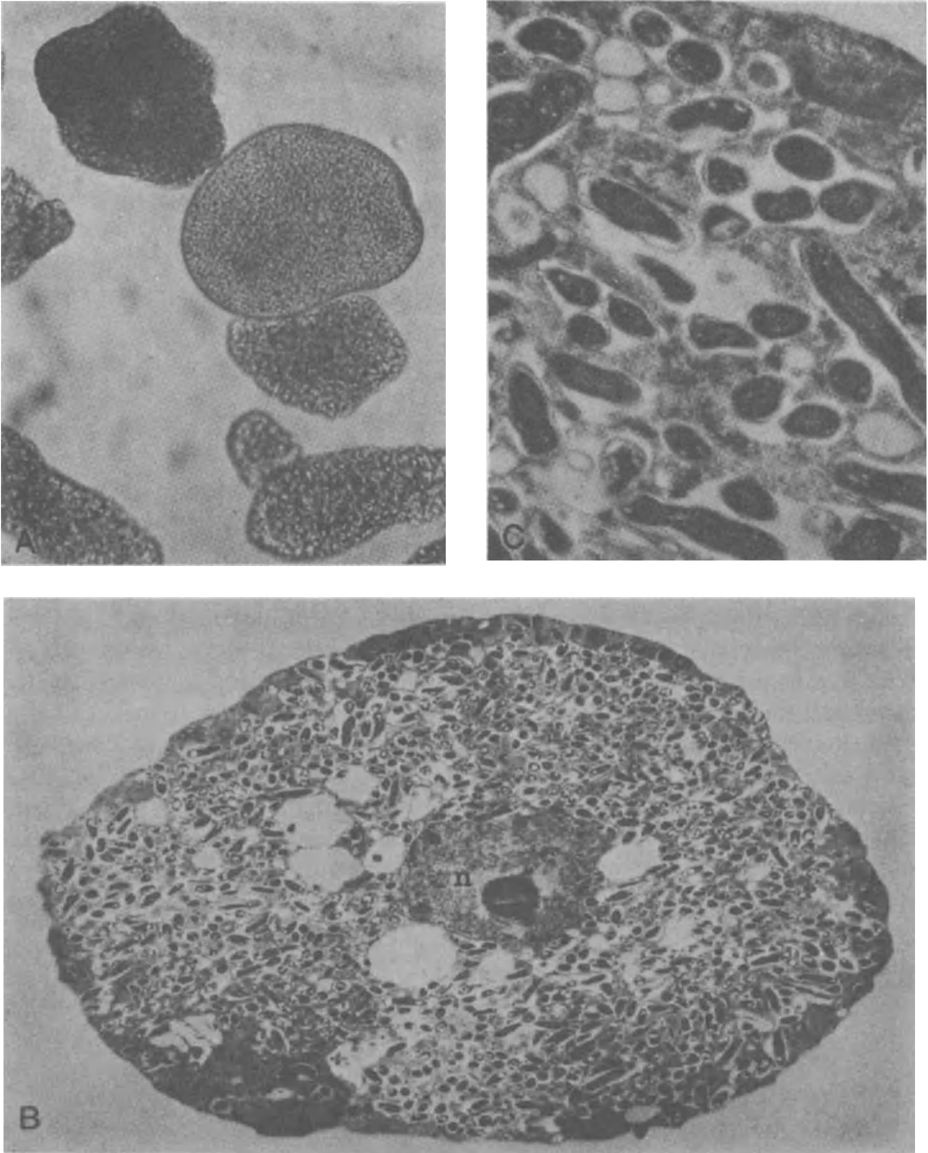

Fig. 2A–C. Isolated soybean root nodule protoplasts. (A) Light micrograph of freshly isolated protoplasts. Bacteroids in the cytoplasm give the protoplasts a granular appearance. × 264. (B) Thin section of a root nodule protoplast showing the large number of bacteroid-containing vesicles in the cytoplasm. *n* = nucleus. × 2400. (C) Bacteroids in membrane-bounded vesicles in the cytoplasm of a nodule protoplast. × 12000

1973; HOFFMANN and HESS, 1973). Evidence also suggests that DNA can be incorporated into higher plant cells (LEDOUX, 1965; LEDOUX *et al.*, 1971a, b; HESS, 1970c, 1972a), while DOY *et al.* (1972, 1973a, b) have reported the transformation of cultured haploid tomato cells by phage DNA. It should be possible in the immediate future to isolate DNA carrying the genes controlling nitrogen fixation from micro-organisms, and to supply this to higher plant cells and protoplasts. The mechanism of uptake of bacterial DNA into higher plant cells and isolated protoplasts is not established; whether it first enters endocytotic vesicles and is then released into the cytoplasm, or whether the genetic material passes directly through the plasmalemma is open to speculation.

11. Summary

Attempts to gain more understanding of nitrogen fixation by symbiotic and free-living organisms have resulted in the development of in vitro cell systems to study the process. Enzymatic digestion of the walls of higher plant cells produces a population of isolated protoplasts. Removal of the barrier normally imposed by the cell wall permits these naked cells to take up particulate materials, including bacterial cells, into membrane-bounded vesicles in their cytoplasm. The uptake of bacteria provides an experimental system with which to study the behaviour of micro-organisms in the cytoplasm of higher plant cells where they are normally unable to penetrate under natural conditions. Recent progress in somatic hybridisation and in the manipulation of genetic material from bacteria also hold great promise in attempts to modify higher plant cells to fix nitrogen. However, it will remain to be seen whether procaryotic DNA can be incorporated and expressed by the higher plant genome, whether transformed cells can be selected from cultures, and whether the character for nitrogen fixation can be transmitted through cell generations.

References see page 563.

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Chapter V

Tissue Culture and Plant Pathology

1. Single Cell Culture, Protoplasts and Plant Viruses

A. C. HILDEBRANDT

1. Introduction

Plant cell and protoplast cultures provide important tools to clarify many details of host cell-pathogen interactions at the cellular level (see HILDEBRANDT, 1958; BRAUN, 1959; HIRTH, 1960; BALL, 1966; BAJAJ and BOPP, 1971; TAKEBE and OTSUKI, 1974). Single cells of bacterial plant pathogens were isolated and established as clones by WRIGHT *et al.* (1930). Subsequently, single cell clones of fungi, animal and higher plants have been successfully established. These single cell clones of higher plants were established from isolated single cells and are to be differentiated from strains of cells. Strains of cells may be derived by plating methods on agar and may result from growth of more than a single isolated cell from suspension cultures comprised of mixtures of single cells and small colonies of cells. These latter strains of cells were not necessarily derived from single cells and can, therefore, be differentiated from true single-cell clones. Single-cell clones have provided the means for pure culture studies for higher plant cells comparable to those used for microorganisms. Several methods have been useful to establish single cell clones of higher plant cells.

2. Establishment of Plant Single-Cell Clones

Early work with pathogenic crown gall bacterial single cells stressed the usefulness of a fermented medium for growth and division of isolated single bacteria. Single bacterial cells transferred directly to a drop of fresh sterile nutrient medium mostly failed to grow. However, if the bacterial cell was placed in a drop of medium in which the bacteria were cultured for 24–72 h and subsequently removed by filtration, then the isolated single cell could be observed microscopically to divide to form a small colony of cells. LADEBERG (1954) subsequently used a gridded microscope slide procedure to make mass single-cell isolations that could be followed microscopically. The first successful higher plant single cell isolations were possible with this background in mind. MUIR *et al.* (1954) used the filter paper raft-nurse culture technique with single cells of marigold and tobacco callus cultures (Fig. 1). Cultures of plant cells in liquid medium on a shaker often fragment and develop as a suspension of single cells and small colonies. Single cells may then be observed in liquid medium in a petri dish under a binocular microscope and picked up with a microspatula or micropipette from the cell suspension in liquid or spread over an agar medium. Several days previous to the

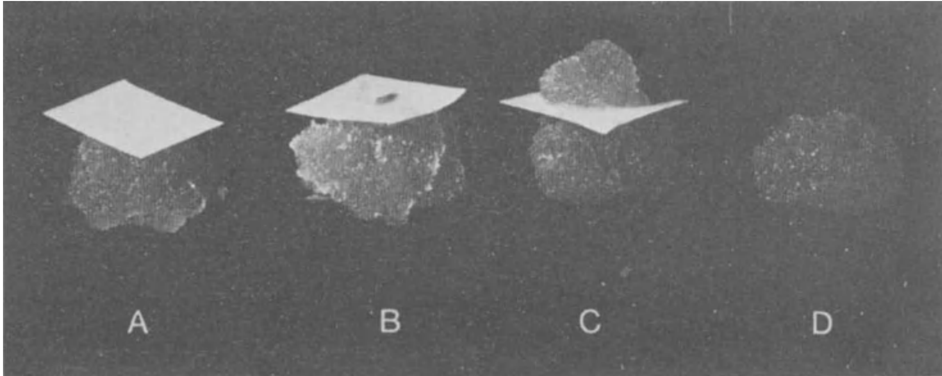

Fig. 1 (A–D). The filter-paper host tissue method of culturing single isolated cells and clones. (A) Established culture of *N. tabacum* × *N. glutinosa* host tissue supporting a filter paper. (B) Filter paper with small colony of cells resting on host tissue. Such a colony developed in several days to weeks from divisions of a hand-picked single cell of *N. tabacum* × *N. glutinosa*. Clonal material of this size usually failed to grow when transferred directly to the basal medium. Such clonal cultures were commonly transferred to several fresh host pieces before they reached the size shown in (C). (D) Established tissue that grew from a single cell as it grew independently on the basal medium following the sequence illustrated in (B) and (C). (HILDEBRANDT and RIKER, 1958)

isolations, sterile 8 × 8 mm squares of filter paper are placed aseptically on top of established callus of the same or different species. The filter paper is wetted by liquid with nutrients from the nurse culture tissue piece. The single cell, then placed on the filter paper raft, may divide and produce a small colony of cells within a few days or weeks. The original single-cell transfer to the filter paper raft should be quickly accomplished to avoid excessive drying of the cell and liquids in the raft. Single cells placed directly on the fresh agar or liquid medium seldom survive, but small colonies of cells that develop on the raft can be transferred directly to fresh medium to continue growth and perpetuation of the single cell clone. TORREY (1957) also showed division of single isolated pea root cells placed around a callus piece on yeast extract medium.

Attempts to isolate and grow single plant cells in hanging drops for long periods (TORREY, 1957) have been less successful, but also should be possible with suitable nutrients and methods that permit sufficient aeration.

Cell plating and cell sieving methods (BERGMANN, 1959, 1960; BLAKELY, 1964; BLAKELY and STEWARD, 1964a, b) have been alternative methods to establish strains and clones of cells. Special microscopic observations and precautions are necessary to insure that a colony of cells originated from a single cell and was thus a true single-cell clone. Cell plating and cell sieving methods have been extensively used and provide an excellent way to isolate quickly large numbers of single cells and colonies.

BERGMANN (1960), with the plating method, followed the repeated divisions and the formation of clones from single cells of *Nicotiana tabacum* L. var. Samsun and *Phaseolus vulgaris* var. Early Golden Cluster. The tissues were grown in

100 ml of liquid modified White's medium (WHITE, 1943) in 250-ml Erlenmeyer flasks on a shaker (120 rpm). These cultures grew as a suspension of single cells and as small clusters of cells. Suspensions of noninjured cells were obtained by two successive filtrations of the cellular suspension under aseptic conditions. To obtain suspensions of single cells, the end of a glass tube was covered with gauze and inserted into a flask partly filled with fresh medium and the cell suspension poured into the tube. Single cells and small colonies of cells passed through the gauze screen into the fresh medium. Using two flasks with gauze of different meshes (0.1 and 0.3 mm width), large cell colonies were separated from single cells and small colonies. A second filtration through the fine mesh gauze separated the single cells from small cell colonies. Over 90% of the cells in such filtrations consisted of single cells. The single cells were then isolated by plating the suspension on White's agar medium in petri dishes or within the melted and cooled agar in the petri dishes. The dishes were then sealed with rubber bands to prevent excessive drying of the agar and incubated at 22° C in diffuse light. With the agar layer, 1-mm thick cells could be observed through the bottoms of the dishes with an inverted microscope. The first cell divisions occurred between the second and fourth days after plating. This method permits the screening of large numbers of cells for genetic and other studies. REINERT and VON ARDENNE (1964) used a plating technique to isolate single cells from established tissue cultures of *Vitis vinifera*. Division of the isolated single cells occurred only in the presence of living tissue fragments and cell groups as had been reported earlier by other workers with single cells. They obtained growth with their plating technique of 60–70% of the single cells.

The cell sieving and plating methods were also used effectively by BLAKELY (1964) and BLAKELY and STEWARD (1964a, b) to study variations in single cell strains of tissues derived from long established cultures of *Haplopappus gracilis* and *Daucus carota*. Using the plating method, a 3-week-old liquid medium suspension culture was passed through either a sterile cheesecloth filter or a fine filter of 173 mesh silk bolting cloth draped over a beaker. The suspension was used directly or concentrated by centrifugation at 100 g for 5 min. One-ml aliquots were removed with a sterile pipette from the filtered suspension and added to 9-ml aliquots of fresh 0.5% agar cooled to 40° C. The 10-ml mixture of medium and cells was poured in a petri dish immediately after mixing. After the medium had solidified, the dishes were sealed with tape and incubated at 21° C in diffuse light. Carrot cells were plated in White's medium with 10% coconut milk, 200 mg/l casein hydrolysate and *Haplopappus* cells were plated in the same medium plus 0.5 mg/l alpha naphthalene acetic acid (NAA) in addition. Carrot cell suspensions filtered through cheesecloth yielded approximately 80% single cells, while suspensions filtered through bolting cloth yielded 90% single cells. *Haplopappus* suspensions filtered through cheesecloth yielded 25% single cells and through bolting cloth 30% single cells. With the "poured-plate" method, the cells were evenly distributed over the plate. Easily visible colonies began to appear on the plates within 2 weeks. The most effective medium for growth of the carrot single cells and colonies of cells contained coconut milk and casein hydrolysate and the *Haplopappus* cell medium NAA additionally. Media were routinely sterilized in the autoclave.

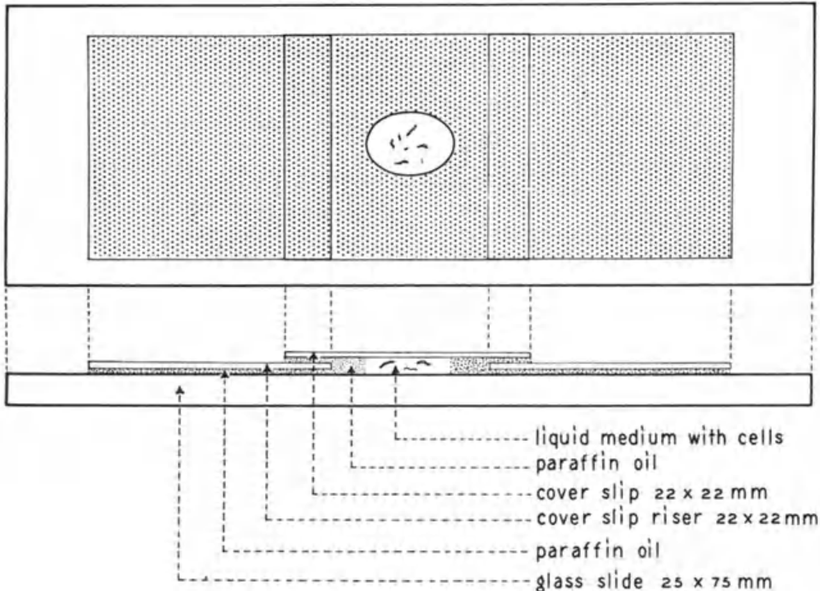

Fig. 2. Microculture chamber. Single cells or small groups of cells remain alive for months in a sterile drop of medium and their growth, division and senescence can be observed. (JONES *et al.*, 1960)

An additional, especially useful method of isolating and establishing single-cell clones of higher plant cells is the microscope slide culture (microculture) technique. With this method, the single cell is cultured aseptically in a drop of liquid medium surrounded by sterile mineral oil on a microscope slide (Fig. 2). A drop of liquid suspension culture is either removed from the culture medium directly with a sterile micropipette, or the suspension poured into a sterile petri plate and a single cell picked up with the micropipette while viewing the suspension using a binocular microscope. The drop of medium containing the selected cell or cells is placed on a sterile microscope slide and ringed with sterile mineral oil. A drop of mineral oil is placed on each side of this drop and a sterile coverslip placed on each drop of mineral oil. A third coverslip is then placed across the culture medium and single cell and bridging the two side coverslips, thus providing a microchamber to enclose the single cell aseptically within the mineral oil boundary. The microculture chamber is then placed in a petri dish and incubated. Cells may live and grow for days and months in the single drop of nutrient without changing the nutrient. The mineral oil prevents water loss from the microdrop, but permits exchange of oxygen. If a colony of cells becomes sufficiently large, the coverslip may be raised aseptically and the colony of cells transferred to fresh liquid or agar medium. This method was originally developed by Jones *et al.* (1960) to observe cytological details of living, unstained tobacco cells as they grew, divided and matured. However, this simple, inexpensive slide culture technique has proved a valuable method to examine many details of host–parasite interactions with phase and interference microscopy (Fig. 3).

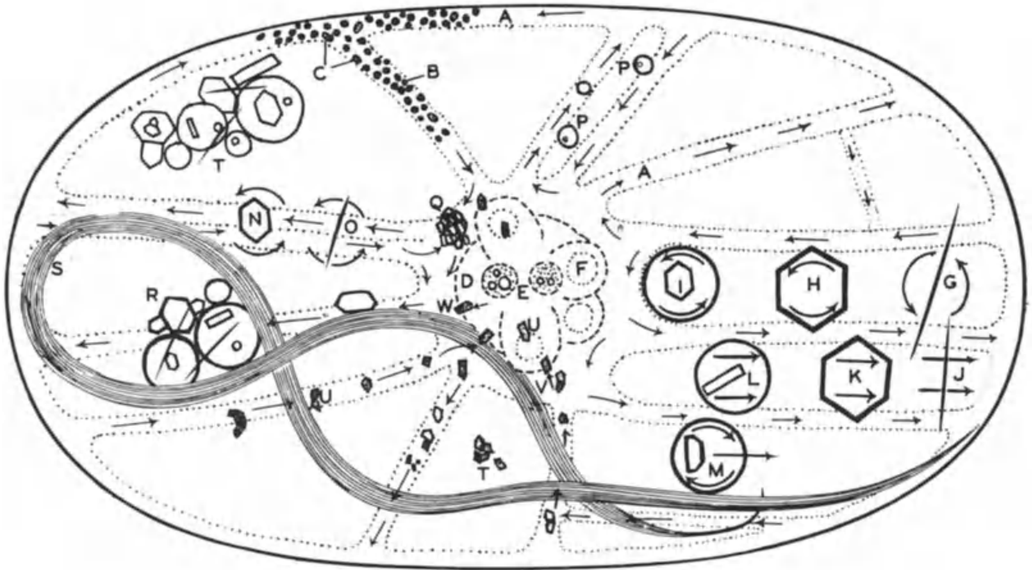

Fig. 3. Diagrammatic illustration of a tobacco cell infected with tobacco mosaic virus as observed through still and cinephotomicrography, showing the morphology, shape and the movement of inclusion bodies; crystals, spherical bodies, fibrous needles, and their aggregates appearing in the cytoplasm. Peripheral and transvacuolar cytoplasmic strands *A* with spherosomes *B* and mitochondria *C*. *Arrows in the strands:* direction of streaming. *Arrows within or about the inclusions:* direction of movement. The centrally placed nucleus *D* has two nucleoli with vacuoles *E* and several lobes with vacuoles *F*. A needle *G*, a crystal *H*, and a spherical body *I* rotate between adjacent cytoplasmic strands streaming in opposite directions. Linear movement of inclusions *J*, *K*, and *L* occurs between adjacent strands flowing in same direction. A spherical inclusion *M* shows both the rotatory and linear movements. A crystal and needle *N* and *O* are rotated by two adjacent but oppositely streaming strands. Small inclusions *P* move in a single strand. They may return toward the periphery of the cell before reaching the nuclear region because of contact with the adjacent and oppositely flowing strand. A group of angular virus crystals *Q* aggregated in close proximity to the nucleus. The cytoplasm moves in various directions from and to the converging and diverging strands, respectively. The angular virus inclusions in a group under a constant stress of cyclotic forces may either break up into individual inclusions or undergo an orientation to form a definite shape of a hexagonal crystal. Movement of several small to large inclusions *R* is obstructed by a loop of a bundle of long fibrous needles *S*. Inclusions *T* not in contact with the streaming strands show little or no movement. Small inclusions *U* are seen in the nucleus and cytoplasmic strands. The inclusions appear to enter *V* and leave *W* through the nuclear membrane. (SINGH and HILDEBRANDT, 1966)

Microscopic observations of living single cells and small colonies of cells in slide microcultures have revealed variations in cell shapes (REINERT, 1956; KOHLENBACH, 1959; JONES *et al.*, 1960; JOSHI and BALL, 1968; KANT and HILDEBRANDT, 1969). Subsequent nutritional studies have indicated considerable variations may also exist in the nutritional requirements (HILDEBRANDT, 1958; HILDEBRANDT and RIKER, 1958; REINERT, 1963; BLAKELY and STEWARD, 1964a,b; REINERT and von ARDENNE, 1964; Fig. 4) and growth habits of single-cell clones derived from a mass culture of cells or of secondary single-cell clones obtained from a selection of cells from a single-cell parent clone (SIEVERT and HILDEBRANDT, 1965).

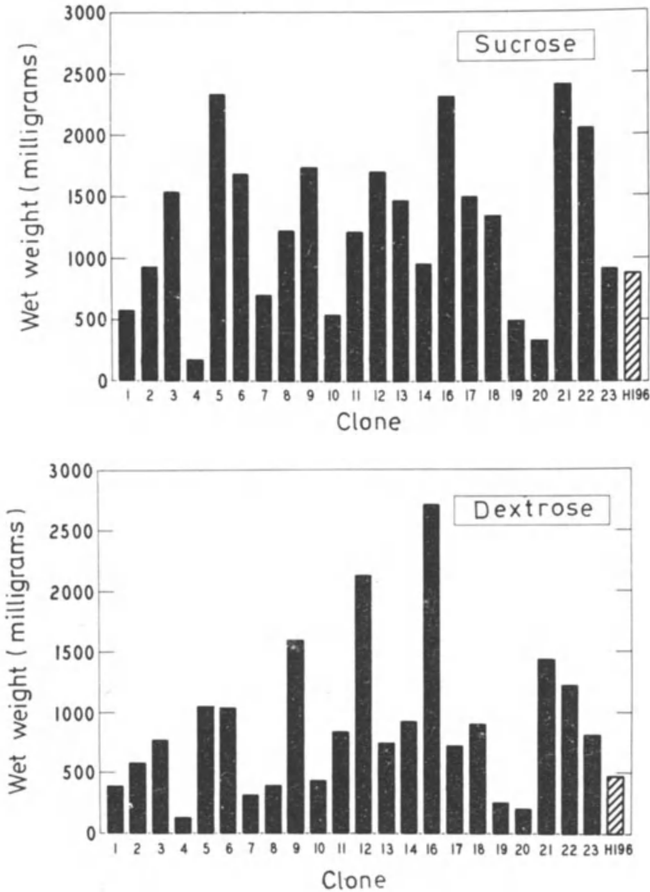

Fig. 4. Average wet weight per tissue piece of the parent single-cell clone (H 196) and 23 secondary single-cell clones of H 196 after 5 weeks on casein hydrolysate medium with 2% of sucrose or dextrose. (SIEVERT and HILDEBRANDT, 1965)

Most of these studies of single-cell clones and secondary single-cell clones have been carried out with tobacco cells and may not necessarily be similar to single-cell clones from other species. It has been, for example, quite evident from published and unpublished studies that certain strains of callus are composed of morphologically uniform cell types, while other strains may consist of a great number of different sized and shaped cells.

3. Applications of Single Cell Clones

Single cell isolations and growth have been possible from many species of higher plants. They have been used to clarify cell growth, differentation, cellular cytological and morphological details, and host cell-pathogen interactions. The filter

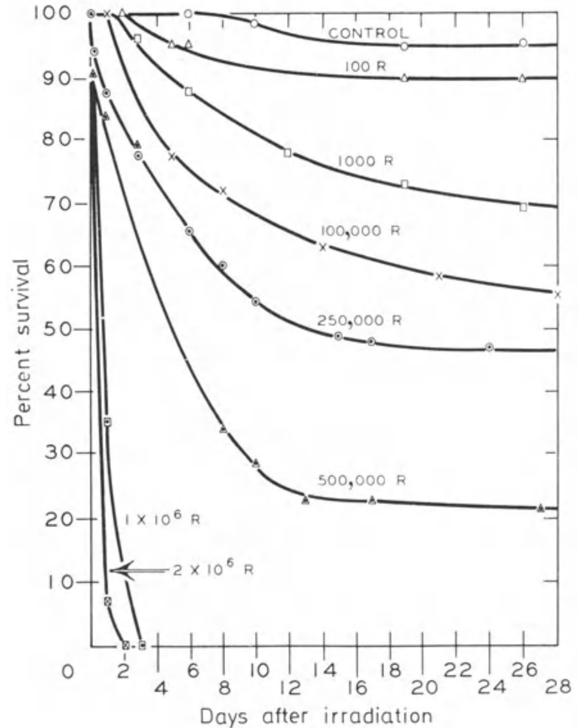

Fig. 5. Viability curves of healthy tobacco cells in microcultures over a period of 28 days after cobalt 60 gamma-radiation doses as indicated. (CRUZ and HILDEBRANDT, 1968)

paper-raft technique was used by BRAUN (1959) to produce plants from single cells of callus from teratomaceous crown galls. Similarly, KALIL and HILDEBRANDT (1971) used the microculture method to show the increased degradation of starch grains in host cells of tomato by crown gall bacteria across a mineral oil barrier separating the pathogenic bacteria from the tomato cells. In this latter case, crown gall bacteria were separated from tomato cells by the mineral oil. Time-lapse motion picture photography was used to show the constant movement of virus and host cell inclusions of tobacco cells growing in microslide cultures (JONES *et al.*, 1960; MOTA *et al.*, 1964; BALL, 1966; DAS *et al.*, 1966; SINGH and HILDEBRANDT, 1966, 1967a, b; NIMS *et al.*, 1967, CHANDRA and HILDEBRANDT, 1967; KANT and HILDEBRANDT, 1969). Gamma radiation induced in healthy and TMV-infected tobacco cells in microculture striking cytological changes, depending on the dosage (CRUZ and HILDEBRANDT, 1968) (Fig. 5). The ability of single tobacco cells to divide, produce a colony of cells and then differentiate plants was also demonstrated starting from single isolated cells in microculture (VASIL and HILDEBRANDT, 1965; CHANDRA and HILDEBRANDT, 1967; Fig. 6).

The tobacco mosaic disease of plants induced by tobacco mosaic virus (TMV) has been studied with isolated cells, tissue and protoplast cultures of tobacco and other species. The early work by WHITE (1934) studied the location of TMV in tomato roots. His assays of the sections of tomato roots showed that the TMV was present in increasing quantities as one took samples increasingly distant from the root tip. The apical 2–3 cm were often free of TMV. These early studies, in

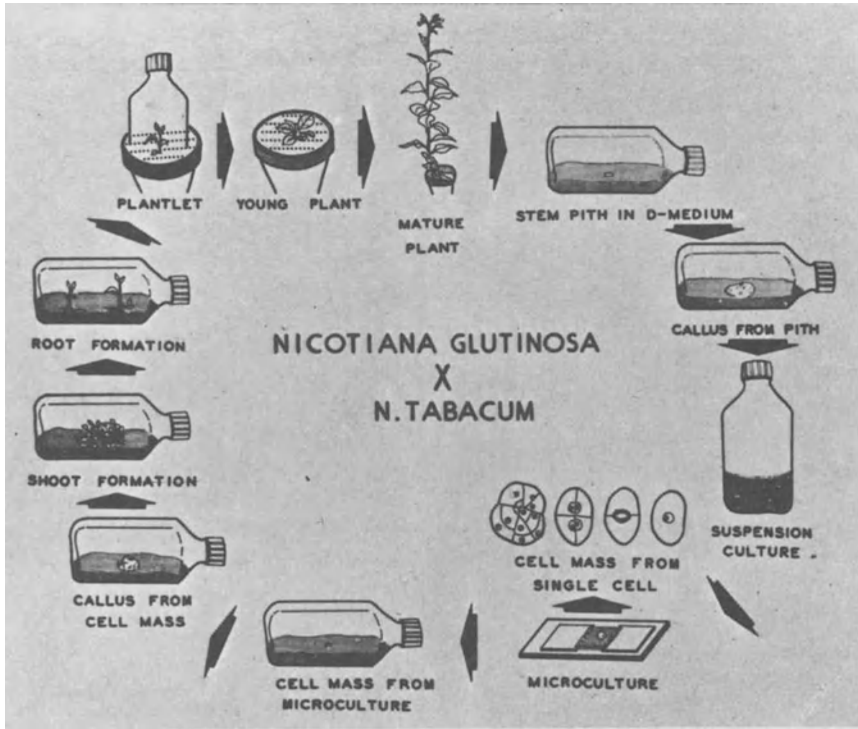

Fig. 6. Diagrammatic representation of the development of normal tobacco plants from isolated, single cells of fresh stem pith callus. (VASIL and HILDEBRANDT, 1967)

retrospect, suggested meristem and callus cultures as possible sources of virus-free plants, although at the time, neither cell nor callus cultures had been established nor the methods perfected for induced plantlet differentiation from callus.

Tomato roots were later artificially infected with TMV in vitro (MELCHERS and BERGMANN, 1957). Similarly, MOREL (1948) showed that undifferentiated tobacco callus infected with TMV could be cultured for unlimited periods in vitro. Various workers (MOREL, 1948; HIRTH and SECRETAIN, 1956; HILDEBRANDT, 1958; BERGMANN, 1959; HIRTH, 1960; HIRTH and DURR, 1971; MURAKISHI *et al.*, 1971) were also able mechanically to inoculate the tobacco callus cultures growing on agar or on liquid medium with the TMV. It is interesting that TMV-infected tobacco callus established by MOREL from TMV-infected stem sections maintained virus replication for at least 20 years through regular monthly transfer periods in this laboratory. On the other hand, established tobacco callus artificially inoculated in liquid or on agar medium often became virus-free after a relatively few monthly subcultures (COOPER *et al.*, 1962; Fig. 7). The reasons for loss of virus are not clear, but have added support to the usefulness of tissue culture methods as means of eliminating virus pathogens from economically important food, fiber and ornamental plants. Some single cells or small colonies of cells in a complex liquid suspension culture or in an

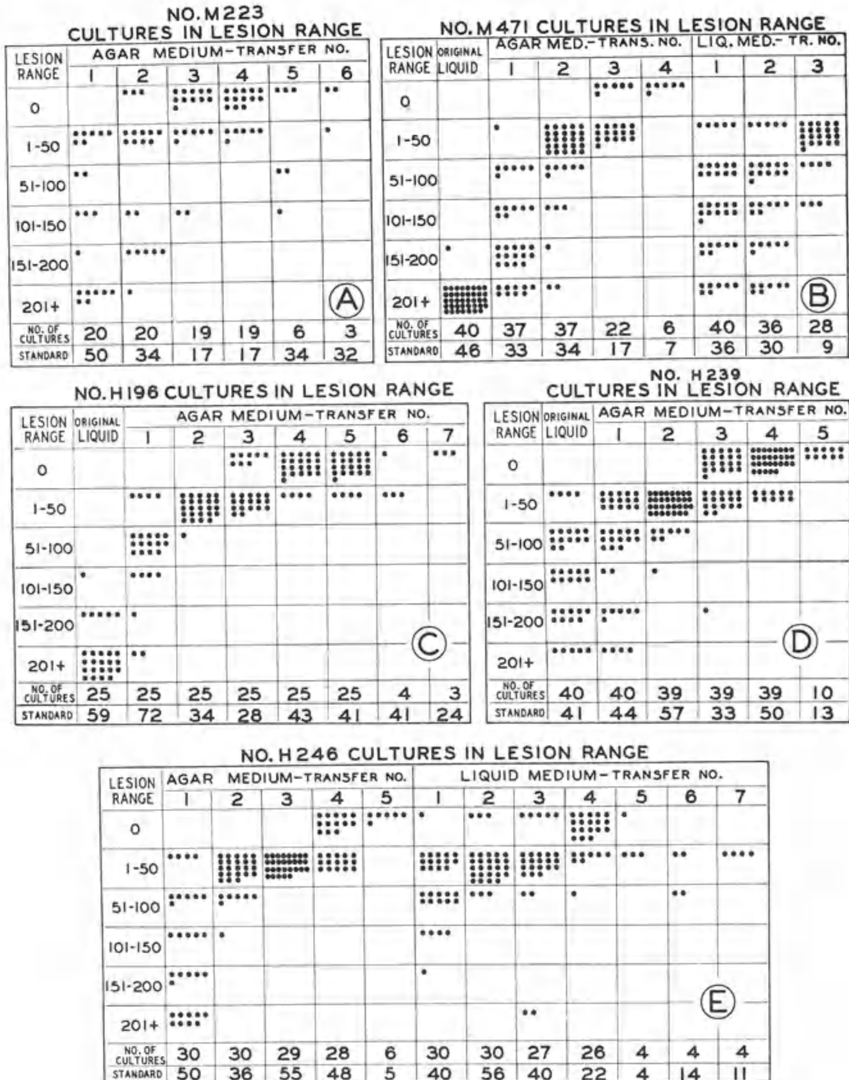

Fig. 7 A–E. Longevity of TMV activities in single-cell clones of tobacco growing in liquid or on agar media. Systemic host (*N. tabacum*) single-cell clones M 223 and M 471 and local lesion host (*N. tabacum* × *N. glutinosa*) clones H 196, H 239 and H 246 were artificially inoculated with TMV. Ranges of virus activities determined on local lesion tobacco assay plants at 1–7 monthly transfer periods as indicated. (COOPER *et al.*, 1962)

agar medium-grown culture are somehow able to escape the virus infection. Cultured tobacco cells were resistant to virus infection at certain stages in their life cycle (WU *et al.*, 1960). One single-cell clone of tobacco tissue selected a mild strain of TMV following artificial inoculation in culture with TMV from TMV-infected tobacco plants (Fig. 8). The tissue culture selected mild strain induced a much milder mosaic in Havana 38 variety than did other strains. The type and

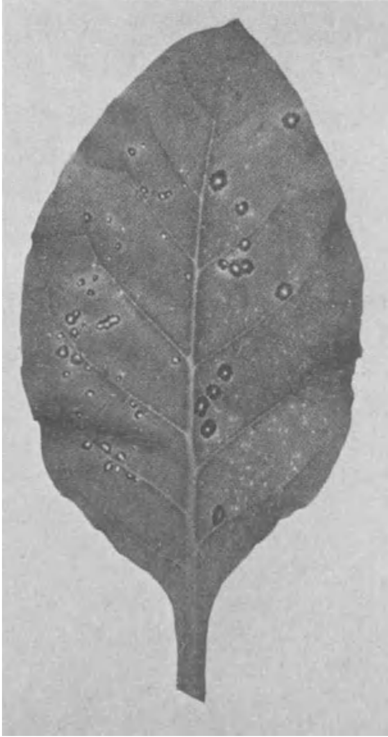

Fig. 8. Lesions induced by three strains of tobacco mosaic virus on the hybrid tobacco leaf. *Left half:* Johnson's mild strain. *Right half:* Johnson's severe strain (large lesions), and very mild strain from tobacco tissue culture (small lesions). All inoculations were made at the same time, and the picture was taken 12 days after inoculation. (WU *et al.*, 1960)

number of injuries produced in the cultured cells by the inoculation procedure may also vary when the cells are artificially inoculated with TMV (KASSANIS *et al.*, 1958).

Other viruses and host tissues have been studied in tissue culture. For example, tobacco mosaic virus was studied in tomato callus (MURAKISHI, 1965) and southern bean mosaic virus has been examined in bean callus (MURAKISHI and PELCHER, 1974). Single-cell clones of tobacco tissue have varied in their susceptibility to TMV infection and multiplication (HILDEBRANDT, 1958; HILDEBRANDT and RIKER, 1958; SAMPATH *et al.*, 1966; CHANDRA and HILDEBRANDT, 1967; HIRTH and DURR, 1971). Nutritional and other environmental conditions under which callus cells were grown influenced the virus activity in the infected cultured cells (HILDEBRANDT, 1973). The TMV activity and the host tissue growth was dependent on the concentration of nitrogen, phosphate and potassium (HILDEBRANDT, 1958). Increased levels of $NaNO_3$ (for nitrogen) from 0.4–25.6 mg/l resulted in increased virus activity over that in control tobacco callus cultures even though at 25.6 mg/l $NaNO_3$ the host tissue cell growth was strongly inhibited (Fig. 9). Increased concentrations of KCl (for potassium) from 0.1–6.4 mg/l also resulted in increased virus activity and host culture growth, but at 25.6 mg/l both virus activity and tissue growth were reduced. Increased levels of NaH_2PO_4 (for phosphate) from 0.003–0.33 mg/l favored host cell growth, but progressively decreased the virus activity in the tis-

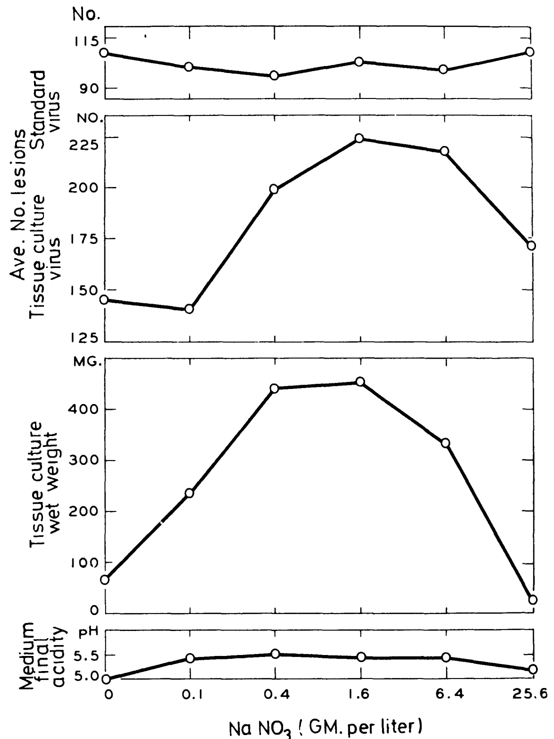

Fig. 9. Effect of increased concentration of $NaNO_3$ on host tissue growth after 6 weeks' incubation expressed as the average wet weight in milligrams. The virus activity is expressed as the average number of local lesions produced in half-leaf, local-lesion assay by a homogenate on a unit-weight basis of the virus-infected tissue from the respective media. The average number of local lesions produced by a dilution of standard TMV on opposite half-leaves is indicated at top. (HILDEBRANDT, 1958)

sues. Varying concentrations of $NaNO_2$ and ammonium compounds also influenced virus activities and culture growth (HILDEBRANDT and RIKER, 1958). Among the nucleic acids and a number of purines and pyrimidines (HILDEBRANDT and RIKER, 1958; WU *et al.*, 1960), the effect of 6-methyl purine (KURTZMAN *et al.*, 1960) was especially interesting in its inhibitory effect on virus activity in the tobacco callus cultures at low concentrations that were stimulatory or only slightly inhibitory to the host cells (Fig. 10). Concentrations of thiouracil and thymine, although they reduced markedly the virus concentration, also inhibited host cell growth as well.

Severe and mild strains of TMV have been inoculated to tobacco cell cultures and subsequently removed from the cultures (HIRTH, 1960; WU *et al.*, 1960). TMV assays were made with local lesion host plants. The TMV activity in single cultured tobacco cells has also been assayed on *N. tabacum* × *N. glutinosa* (HANSEN and HILDEBRANDT, 1966).

All strains and clones of tobacco cell cultures do not respond similarly to TMV infections (HILDEBRANDT and RIKER, 1958; Table 2). Some strains have remained infected with TMV for 10 years or longer (HILDEBRANDT, 1958). Other strains of tobacco cultures lost their virus infectivities after several transfers to agar or liquid media. Tobacco single-cell clones inoculated with TMV and incubated in liquid shake cultures remained infected longer than agar-grown cultures. Certain clones lost the virus infectivity after seven monthly subcultures (COOPER *et al.*, 1962; Fig. 7). The chromosome numbers of cells of two single

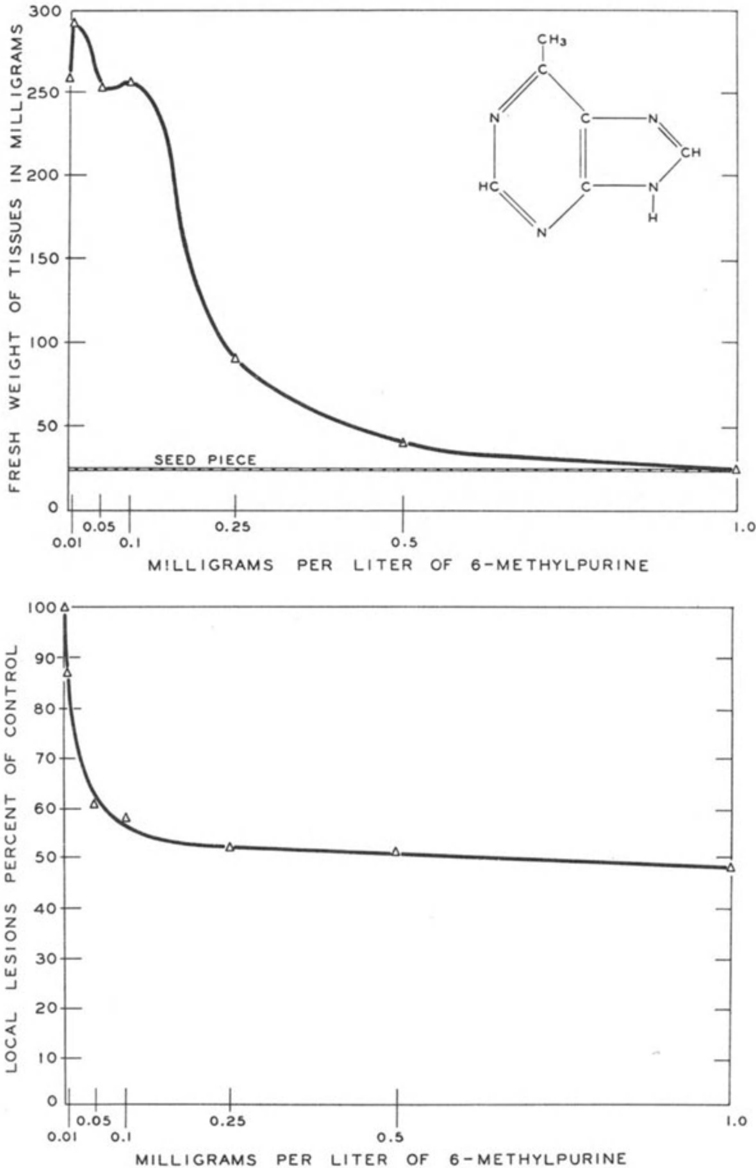

Fig. 10. Effect of 6-methylpurine on growth of MOREL'S tobacco tissue (*top*) and upon the virus concentration in the tissue (*bottom*). (KÜRTZMANN *et al.*, 1960)

cell clones of tobacco (*N. tabacum*) tissue isolated from crown gall tissue origins originally obtained from MOREL have been examined (COOPER *et al.*, 1964). Both clones were grown for eight years on a modified White's medium supplemented with coconut milk and 2,4-dichlorophenoxyaceticacid. Chromosome counts of 110 polar views of metaphase plates revealed chromosome numbers of 48, 96, and 192, the majority having 48 (Figs. 11, 12).

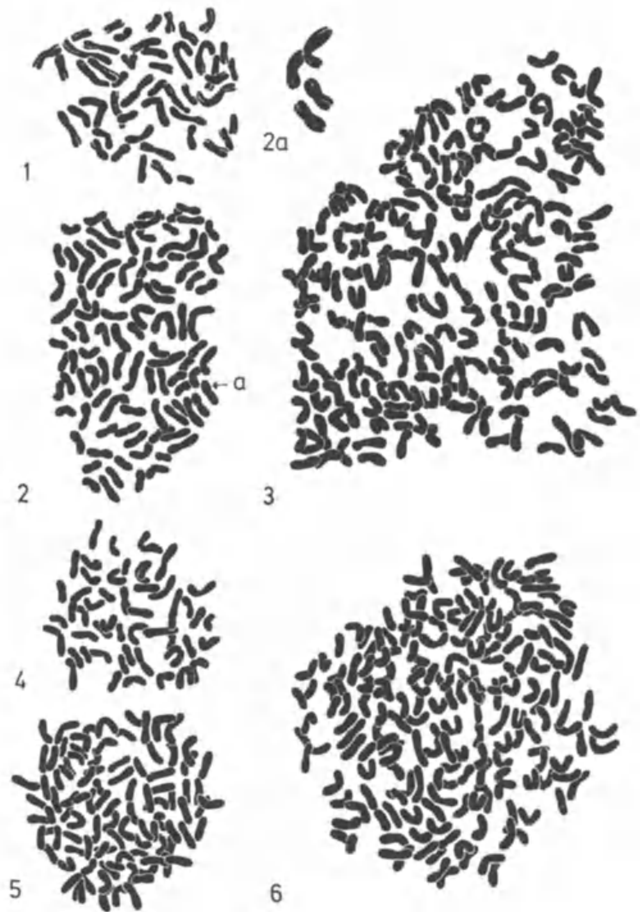

Fig. 11. Camera lucida drawings of polar views of metaphase plates of single-cell tobacco clones M223 and M471 (1–3), M223. (1) $2n=48$. (2) $4n=96$. (2a) 4 chromosomes with 2 chromatids; see arrow. (3) $8n=192$. (4–6) M471. (4) $2n=48$. (5) $4n=96$. (6) $8n=192$. (1–6) ×2010; (2a) ×2950. (COOPER *et al.*, 1964)

The early work with tomato root tips (WHITE, 1934; MELCHERS and BERGMANN, 1957) and later work with single-cell clones and mass cultures of tobacco (HILDEBRANDT, 1958; HILDEBRANDT and RIKER, 1958; WU *et al.*, 1960; SAMPATH *et al.*, 1966; HIRTH and DURR, 1971), for example, have indicated that young, actively growing cells are more resistant to infection with TMV than old cells in older cultures. WU *et al.* (1960) demonstrated this influence of cell age and type on susceptiblity to infection and subsequent multiplication. Tissue cultures of tobacco (*N. tabacum* × *N. glutinosa* single-cell clone H 239) were inoculated with TMV at different stages of cellular development. Young cultures consisting largely of meristematic cells were more resistant to infection and subsequent virus multiplication than the older cultures that contained largely enlarging and old cells (Table 1).

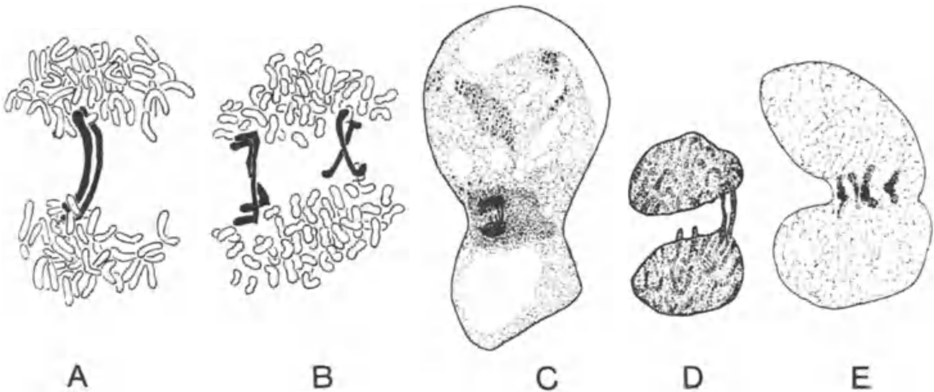

Fig. 12.A–E. Abnormal anaphase and telophase configurations of single-cell tobacco clone M 223 with chromosome bridges leading to polyploidy. (A, B) Anaphases with chromosome bridges. (C) Cell containing a telophase with chromosome bridges. (D) Nuclei of (C) enlarged (E) Early interphase with conjoined nuclei. (C) × 450; all others, × 1840. (COOPER *et al.*, 1964)

Table 1. Tobacco mosaic virus activity in certain representative single-cell clones and host tissue growth

Species and clone no.	Local lesions[a] from		Tissue weight[b] g
	Tissue No.	Medium No.	
N. tabacum			
M 222	0	6	10.39
M 223	0	0	2.05
M 471	359	184	3.74
N. glutinosa			
G 252	83	4	0.54
G 283	146	5	0.40
N. tabacum × *N. glutinosa*			
H 194	158	7	8.56
H 196	265	2	9.55
H 237	77	6	4.10
H 241	4	2	10.66
H 248	5	5	13.66
H 261	0	0	11.90

[a] 1 : 1 dilution of tissue homogenate with distilled water assayed after the 4th monthly transfer in liquid 'D' medium.
[b] Average weight in g of tissues in 4 bottles at the end of one month's incubation. (HILDEBRANDT and RIKER, 1958).

Many other virus pathogens in infected plants also tend to be progressively diminished or lost from cells as the apical stem tip or root tip is approached (BAJAJ and DIONNE, 1966). Thus, aseptic culture of apical meristems has been used to obtain virus-free plants directly or the stem tips were first induced to produce callus and then plants were differentiated from the callus. Early work by MOREL

Table 2. Relationship between culture ages of single-cell clone H239 and to susceptibility to infection and subsequent multiplication by TMV (Johnson's mild strain)[a]

Inoculation, days after transfer	Predominant cell type	Virus assay, days after transfer	Relative infectivity
3	Meristematic	10	0.10
10	Enlarging	17	0.52
17	Enlarging	24	0.42
25	Enlarging	32	0.40
32	Senescent	39	0.77
40	Senescent	47	0.70

[a] From WU *et al.* (1960).

Fig. 13. Vigorous, large-flowered, virus symptomless geranium plant from meristem callus culture *(right)* of commercial virus infected and stunted mother plant *(left)*, and plant *(center)* from 3-inch cutting of plant *(left)*. All plants of comparable age. (After ABO EL-NIL and HILDEBRANDT, 1974a)

and MARTIN (1952) was done with dahlias and later with potatoes (1955). Subsequently, apical meristem techniques have eliminated virus pathogens and improved the quality and productivity of many species including carnations (BAKER and PHILLIPS, 1962; LABRUN-PEREMANS, 1966; STONE, 1968), potato (KUMAR, 1963; SVOBODOVA, 1966; CHANDRA and HILDEBRANDT, 1967; CHRISTENSEN, 1968; STACE-SMITH and MELLOR, 1968a,b; SIMONSEN and HILDEBRANDT, 1972; TAPIO, 1972; WANG and HUANG, 1975), chrysanthemum (HILL, 1968; EARLE and LANGHANS, 1974), pelargonium (PILLAI and HILDEBRANDT, 1969; ABO EL-NIL and HILDEBRANDT, 1971, 1973, 1974a, b, 1976; Fig. 13), orchids (MOREL, 1960, 1964; KIM, 1969), poplar (BERBEE *et al.*, 1972; WINTON, 1970), cassava (BERBEE *et al.*, 1973; KARTHA *et al.*, 1974b), gladiolus (HILDEBRANDT, 1971; SIMONSEN and HILDEBRANDT, 1971) and sugar cane (HEINZ *et al.*, 1969; KRISHNAMURTHI and TLASKAL, 1974). In the case of cassava (BERBEE *et al.*, 1973), heat therapy was used along with apical meristem culture to eliminate two serious virus diseases

(leaf distortion and mosaic) from the plants. Plants of many other species have been differentiated in tissue culture and are discussed in Chap. V. 2,3 of this volume.

4. Plant Viruses and Protoplasts

Further applications of cultures of higher plant cells to plant pathological problems have been emphasized with the development of cultures of naked protoplasts of plant organ or callus tissue origins (TAKEBE *et al.*, 1968; AOKI and TAKEBE, 1969; COCKING, 1970; SCHENK and HILDEBRANDT, 1971; ZAITLIN and BEACHY, 1974, see also Chap. IV.1 of this Vol.). Tobacco mesophyll protoplasts have been extensively studied for various growth activities or for TMV virus-protoplast interactions (COCKING and POJNAR, 1969; COCKING, 1970; NAGATA and TAKEBE, 1970; TAKEBE *et al.*, 1971; TAKEBE and NAGATA, 1973; OTSUKI *et al.*, 1972). The details of the history of protoplasts cultures, the enzymatic removal of the cell walls to free the naked protoplasts, fusion of protoplasts, regeneration of new cell walls, methods of virus transmission and assay and regeneration of plants from protoplasts cultures have been described in the above references and discussed in chapter IV of this volume. The preparation of protoplasts from leaves or other organs requires special aseptic techniques to insure that the protoplast cultures are sterile. Sterile callus cultures have certain advantages over intact plant protoplasts as a source of sterile protoplasts.

Protoplast cultures, in addition to their potential values for genetic, physiological and somatic hybridization studies, offer many possibilities to clarify additional host cell-pathogen reactions at the cellular level (TAKEBE *et al.*, 1968). The plant pathologist, for example, sees this technological breakthrough in cultures of protoplasts as a means to extend studies to viruses other than TMV and other pathogens that are not easily transmitted to whole plants for various unknown reasons. The cell wall is often a major barrier to virus penetration by mechanical rubbing techniques. Insects or nematodes, for example, may be necessary as vectors for transmission of a variety of important viruses to whole cells. The naked protoplasts without the mechanical barrier of the cellulose wall thus provide an important tool to study virus transmission and subsequent infection and virus replication. In addition to plant protoplast-virus studies referred to earlier, efforts have been made to utilize the techniques with other viruses and protoplasts from other species including cucumber mosaic virus, potato viruses X and Y, pea enation virus and cowpea mottle virus (COUTTS, 1972; ZAITLIN and BEACHY, 1974). The TMV multiplication in cultured protoplasts is comparable to that in infected growing plants and a high percentage of the protoplasts are infected with virus (COUTTS *et al.*, 1972; COUTTS, 1973). The procedure of using protoplasts for virus studies has been extended by TAKEBE and OTSUKI (1974) to experiments with TMV and cucumber mosaic virus (CMV), and it was indicated that both viruses could be transmitted to individual protoplasts. The ultrastructure of tobacco mesophyll protoplasts infected with TMV (HIBI and YORA, 1972; HONDA *et al.*, 1973) and cucumber mosaic virus (HONDA *et al.*, 1973) has also been described.

The infection of tobacco protoplasts with cowpea chlorotic mottle virus and its RNA (MOTOYOSHI *et al.*, 1973) and TMV-RNA (AOKI and TAKEBE, 1969) has been possible. Virus replication in protoplasts has been assayed by local lesion assays on suitable host plant leaves and by fluorescent antibody staining (OTSUKI and TAKEBE, 1969; MOTOYOSHI *et al.*, 1973). It is anticipated that protoplast-pathogen interactions will be further clarified with this technology and with increasing numbers of viruses, other pathogens and metabolites currently difficult to study with whole cells and whole plants.

5. Conclusions

It is possible to isolate and grow single plant cells of many species by sterile culture methods. In most cases, either a nurse culture or neighboring cells are necessary for the continued growth and division of the isolated single cell. In certain cases, a conditioned medium (one in which cells have been growing several days or weeks, but then filtered off) is essential and beneficial for growth of the single cell. Cell plating and especially the microculture method are useful for continued microscopic observation of cell growth and division. Virus inoculations of cells and colonies of cells in liquid or on agar media may be accomplished with a number of different plant viruses. Virus multiplication within the inoculated cells may be demonstrated with local lesion host assays and with serological methods. Certain viruses have not been possible to transmit to the intact cells perhaps because of the cell wall barrier to virus penetration. Protoplasts from which the cell walls have been enzymatically removed provide a major advantage to study virus-host protoplast interactions and to understand further virus replication.

References see page 636.

2. Meristem Culture and Virus-Free Plants

F. QUAK

1. Introduction

Virus multiplication is so intimately associated with normal metabolic processes in plants that selectively interfering with it may not be possible at all. Known virus inhibitors have proved toxic to the plant. Moreover, they cannot eliminate virus from a whole plant: when the treatment ceases, the virus soon recovers to its former concentration.

Killing vectors, such as insects, nematodes and mites, may alleviate the spread of certain virus diseases. Some viruses, however, are spread mechanically, others are stylet-borne, which means that they are transmitted immediately the insect starts feeding. Such viruses cannot be controlled by pesticides.

Fortunately the majority of known viruses are not transmitted by seed, and the seeds of infected plants usually develop into healthy plants. However, seedlings are useless for maintenance of clones, hybrids and varieties. Once systemically infected, the disease is transmitted from one vegetative generation to the next, which over the years has led to many varieties of various crops becoming completely infected. Probably all clonal crops are infected with one or more viruses, particularly with latent viruses which are hardly detectable by their symptoms.

Occasionally one or more plants of a variety without virus can be selected. In case of latent viruses, routine testing is needed, serologically, with indicator plants or with electron microscopy. For those clones or cultivars found to be fully infected, methods have been developed for freeing them of one or more viruses: heat treatment or meristem culture or a combination of both.

The term virus-free will be used in the limited sense of free from the viruses for which the plant is tested while the unknown virus may remain. In fact new viruses are sometimes discovered in material freed from known viruses. The material concerned should be called "virus-tested" rather than "virus-free". Cured plants are not immune: they may be reinfected.

The success of therapy depends on the virus to be eliminated and on characteristics of the plant material. Heat treatment is usually effective only against isometric and thread-like viruses and against diseases now known to be caused by mycoplasmas.

The four stages in therapeutic operations are: (1) Identification of virus(es) present in the clone. (2) Therapy. (3) Testing of treated plants. (4) Propagation and continued testing of cured plants under conditions that avoid reinfection.

2. Heat Treatment

In 1889 it was noticed in Java that sugarcane suffering from sereh disease (caused by a virus) grew better after having been kept at 50–52° C in water for 30 min (KOBUS, 1890). It is also a means against ratoon stunt, a bacterial disorder. In many parts of the world, several thousand tons of sugarcane cuttings are treated each year in large water baths before being planted (GILLASPIE *et al.*, 1975).

In 1936 KUNKEL found that peach trees could be cured from yellows by a warm water treatment of dormant trees at 50° C for 10 min. He also discovered that the results were actually better if growing peach trees were kept for 2–4 weeks in air at 35–38° C. In fact warm water treatment in general is much more injurious to plants or dormant parts of plants than is warm air. The viruses involved, however, all caused diseases of the yellows type, which are now known to be related with and probably caused by mycoplasmas (DOI *et al.*, 1967; DAVIS and WHITCOMB, 1971).

Later, KASSANIS (1950) was the first to prove that a true virus, i.e. potato leaf-roll virus, can be heat-inactivated in tubers. Since then a stream of papers on the subject has been published. In their review NYLAND and GOHEEN (1969) reported some 90 viruses and 30 diseases of the yellows type to be curable by heat.

Unlike the warm water treatment of sugarcane cuttings, the warm air treatment is mainly used for establishing a virus-free nuclear stock, which is then propagated under conditions preventing re-infection.

As KASSANIS (1965) pointed out, the ability of viruses to infect and multiply in plants at 36° C is not correlated with their thermal inactivation points. Tomato bushy stunt virus, for instance, has a thermal inactivation point of 80° C, but it cannot infect plants at 36° C and it is eliminated from fully-infected plants kept at this temperature. Thus virus elimination by prolonged exposure of infected plants to temperatures around 37° C must be attributed either to some metabolic system which shifts the balance between virus synthesis and virus breakdown in the plant, or to failure of the virus to multiply at this temperature.

In general the viruses which can be eliminated by heat are the isometric ones. Two non-isometric viruses known to be eliminated by heat treatment are apple chlorotic leafspot virus, a filamentous rod (CAMPBELL, 1962), and plum pox virus (KEGLER, 1967). So far rigid rods of the tobacco mosaic virus type have withstood heat treatment.

It was found that not all viruses in a plant react similarly to heat treatment. For the elimination of certain viruses a period of some weeks may be sufficient, whereas for others in the same plant and under the same conditions it may take a much longer period—perhaps even longer than the plant can withstand, as in raspberries (CONVERSE, 1966, 1970), apple (CAMPBELL and BEST, 1964) and carnations (DUNEZ and MONSON, 1968). Even strains of one virus may respond differently to heat treatment.

In some instances the entire plant is subjected to temperatures between 35 and 40° C for periods varying between a few days to several months. This is mainly

applied to strawberries (POSNETTE and CROPLEY, 1958), simply because they don't produce shoots, and to raspberries (CHAMBERS, 1961) in which the canes die in the second year after bearing fruits, so that they cannot be used to graft treated tips. More commonly viruses are eliminated from shoot tips taken from growing plants, and kept at temperatures around 37° C for varying periods. After a sufficiently long treatment the tips are rooted or grafted onto virus-free rootstocks (seedlings). This method has been successfully used to free varieties of various perennial crops from a number of viruses. Particularly with plants that can easily be tip rooted or tip grafted, it is more effective than the previous method (VAN DER MEER, 1975). Thus rootstocks as well as varieties of apple and pear were obtained in which none of the known viruses can be demonstrated.

It is generally accepted that heat treatment does not inactivate the virus, but prevents it from invading shoots developing during treatment. WELSH and NYLAND (1965), however, noticed that several dormant axillary buds of some varieties also lost their virus, suggesting that virus inactivation may not be excluded.

Success of heat treatment is largely determined by the changes of survival of the treated material, therefore the physiological condition of the material is very important. While BAKER (1962) emphasized that for a warm water treatment at 50° C a physiologically dry condition is favorable for plant survival, VAN DER MEER (1967) stresses this point among others for warm-air treatment: it can best be started with material that terminated its growth in autumn and therefore is physiologically drier than it would be in spring. Thus the dormancy of apple and pear trees, well established in pots and somewhat pot bound, placed at 37–38° C with 16 h illumination per day, is broken, and growth is slowly resumed. Material thus treated withstands prolonged heat treatment and shows a high degree of virus elimination. Some workers have also reported that fluctuating temperatures are more favorable for plant survival than constant temperatures (MELLOR and FITZPATRICK, 1961; LARSEN, 1974).

Heat therapy has proved a highly successful method for inactivation or elimination of viruses from perennial crop plants in order to obtain virus-free (virus-tested) stock material. It fails in certain instances but may then provide a valuable pre-treatment of material to be submitted to meristem-tip culture.

3. Meristem-Tip Culture

3.1 Introduction

As early as 1922, KOTTE and ROBBINS independently observed growth of root tips on mineral solutions supplemented with sugars, aspargin, and pepton. Later, WHITE (1943) was able to subculture TMV-infected tomato roots in vitro. Dissecting such roots, and testing the various zones by inoculation of a local lesion host of this virus, he noticed that the virus concentration in the terminal parts was low as compared to that of basal parts and in the root tip he found no evidence of virus at all. Likewise LIMASSET and CORNUET (1949) observed that in systemically infected plants virus concentrations decreased as they approached the vegetation

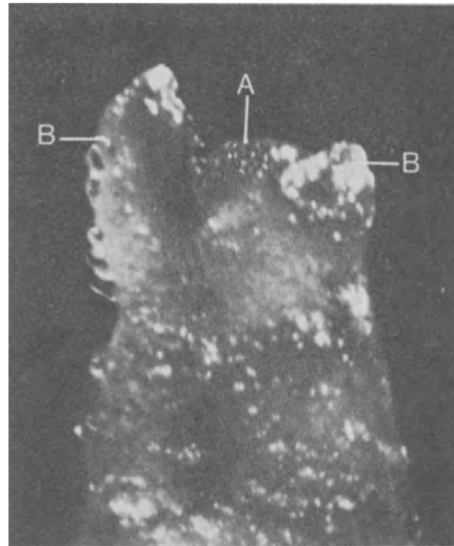

Fig. 1. A growing point of potato with meristem about 0.1 mm in diameter *A*, and two leaf primordia *B* at the time of culture

point (apical meristem). In the vegetation point itself no virus was detectable in half of the cases. This led MOREL and MARTIN (1952) to postulate that it might be possible to isolate the apical meristem of a systemically infected plant in vitro in order to obtain virus-free plants, genetically identical to the "mother plant". They succeeded in confirming this hypothesis by freeing dahlia from viruses. Some of the excised meristems placed on various nutrient media, developed into plantlets of 1–2 cm length, albeit without roots. By grafting them onto young, virus-free seedlings they obtained healthy plants. Ever since this pioneer work, the technique has been used by many workers to cure infected valuable cultivars of a wide range of plants.

The meristem is a dome of actively dividing cells, about 0.1 mm in diameter and 0.25 mm long. Reviewing the literature one is struck by the disparity of terminology. Besides "meristem culture", the terms "meristem-tip culture", "tip culture", "culture of shoot apices" or "shoot tip culture" are used, partly depending on the actual size of the isolated pieces of tissue. As a rule meristem of both main shoot and axillary buds are excised to the dimensions mentioned above. However, the chances of growth are often so little that with the meristem one or two leaf primordia are also excised (Fig. 1). Such tips are up to 1 mm long, and have better chances of developing but are less likely to be free from virus.

3.2 Technique and Medium

Meristems, either apical or axillary are protected by developing leaves and by scales to such an extent that disinfection may be superfluous. For mild surface-sterilization of shoots and defoliated segments of stems they are dipped for a few seconds in 96% ethanol and then submerged in a filtered solution of 50 g/l commercial calcium hypochlorite for 10–20 min and rinsed several times in sterile

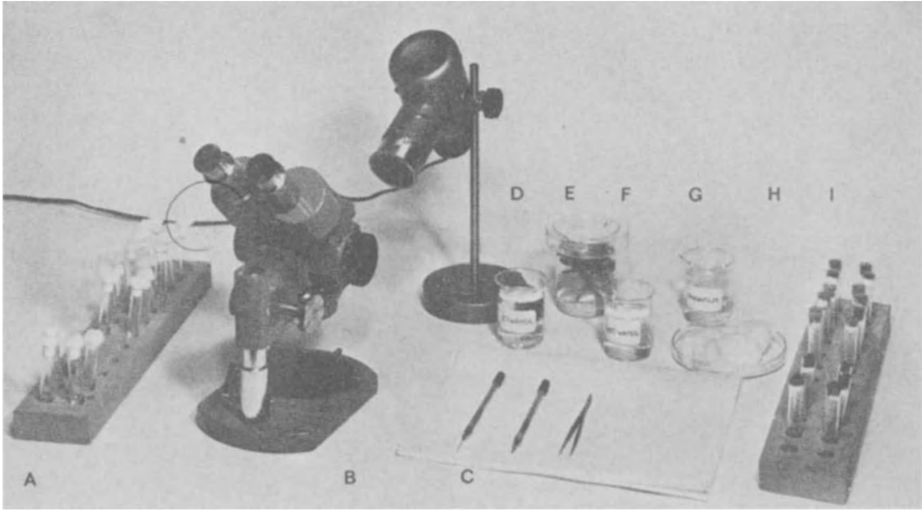

Fig. 2. Equipment needed for excising meristems: nutrient medium in tubes *A*, microscope (× 10–40) and lamp *B*, sterile filter paper, mounted needle, mounted fragment of razor blade and tweezers *C*, ethanol *D*, disinfected shoots *E*, sterile water *F*, Parafilm, disinfected in ethanol *G*, ethanol-soaked cotton wool *H*, and tubes flamed and sealed with sterile Parafilm *I*

water. If no transfer room as used for mycological work is available, the excision of the meristems may be carried out in normal dust-free laboratory space. However, it is highly desirable that the place should be sprayed with ethanol before use. A binocular dissecting microscope (magnification 20–40) is required. The stage of the microscope is wiped with cotton wool dipped in ethanol after isolating each meristem.

On one side of the microscope a package of six to ten sheets of sterile (autoclaved) filter paper and two 100 ml beakers are placed. One beaker is filled with ethanol and the other with sterile water in which dissecting instruments are dipped before blotting them between the sheets of filter paper (Fig. 2). To avoid any microbial and viral contamination, frequent disinfection of the tips of the mounted needles and fragments of safety razor blades with ethanol is essential. Holding the shoot in one hand under the microscope the immature leaves and leaf primordia are snapped off with slight pressure from the needle held in the other hand. The exposed meristem-tip which appears as a shiny dome is then severed with the blade. In some species, such as chrysanthemum, the meristem is flat and including primordia in the excision is unavoidable. The explant is transferred to a nutrient medium in a culture tube by sticking the blade in the agar and withdrawing it slowly. In liquid media the meristems are supported on a filter paper bridge, partly immersed in liquid (GOODWIN, 1966). For detailed technique also see Chap. V.3. of this Volume.

Culture tubes of various shapes and sizes have been used. Particularly for narrow tubes (approx. 10 mm diameter) Pyrex (boronsilicate) glass is recommended. The tubes are sealed with plastic or aluminum caps. Cotton wool plugs

Table 1. Basic medium for meristem-tip culture according to MOREL and MULLER, 1964

Major salts	mg/l
$Ca(NO_3)_2 \cdot 4H_2O$	500
KNO_3	125
$MgSO_4 \cdot 7H_2O$	125
KH_2PO_4	125
KCl	1000
$(NH_4)_2SO_4$	1000
Minor elements	
$FeCl_3 \cdot 6H_2O$	1
$ZnSO_4 \cdot 4H_2O$	1
H_3BO_3	1
$MnSO_4 \cdot 4H_2O$	0.1
$CuSO_4 \cdot 5H_2O$	0.03
$AlCl_3$	0.03
$NiCl_2 \cdot 6H_2O$	0.03
KI	0.01
Organic constituents I	
Gibberellic acid	0.1 mg/l
Sucrose	20 g/l
Difco agar	6 g/l
Organic constituents II (alternative)	mg/l
Myo-inositol	100
Ca-pantothenate	1
Nicotinic acid	1
Pyridoxine HCl	1
Thiamine HCl	1
α-Naphthalene acetic acid	1
Gibberellic acid	0.1
Biotin	0.01
Sucrose	20 g/l
Difco agar	6 g/l

may be flamed, then pushed down under the rim before being sealed with Parafilm. This effectively closes the tubes and prevents dessication for up to twelve months. With flaming cotton wool plugs care must be taken not to burn them all through, as fumes entering the tube have proved toxic to the excised meristem.

In the beginning of meristem culture the nutrient medium was based on that developed by WHITE (1943), to which minor elements were added according to BERTHELOT (1934) or HELLER (1953). Since then many improvements in the media have been made. A useful basic medium is the one devised by MURASHIGE and SKOOG (1962). It is, among other things, characterized by high concentrations of potassium and ammonium ions and of meso-inositol. Increased concentrations were found to be critical for the culture of meristem tips of potato, which developed into plantlets greener and more vigorous than on the previously used lower concentrations of macro-elements (MOREL and MULLER, 1964, see Table 1). Iron is applied in various forms of which iron-chelate complex appears to be the best (MURASHIGE and SKOOG, 1962). As a source of carbon, glucose, fructose or sucrose are available, of which the latter is most widely used.

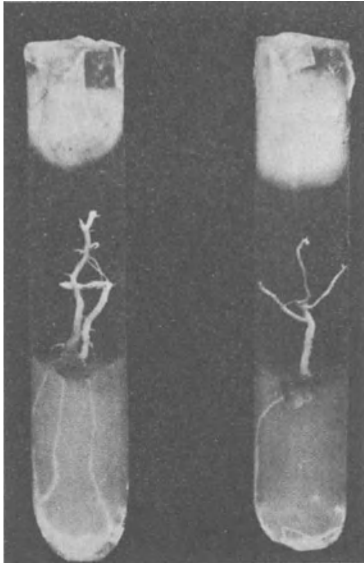

Fig. 3. Potato plantlets obtained from meristem cultures (about natural size) cultured on agar-solidified medium

Besides a mixture of vitamins, a variety of growth substances have been studied for their effects. Although auxins α-naphthalene acetic acid (α-NAA) and 3-indole acetic acid (IAA) are known to stimulate root initiation, their continued use may inhibit root development, and make transfer of the meristem tip to medium without these growth substances necessary. Alternatively, NISHIZAWA and NISHI (1966) kept the sprouts of lily plants for 24 h in a 20 mg/l solution of α-NAA prior to excision of the meristem tip, thus benefitting by a temporary effect of the auxin and avoiding later toxicity.

The effect of gibberellic acid was found to be particularly effective (MOREL *et al.*, 1968). It suppresses unorganized divisions which lead to formation of callus and stimulates the meristem tip to differentiated, often fast growth. Occasionally, as in carnation (QUAK, unpubl.), root development fails under these conditions, even if α-NAA is available, so that transfer to a similar medium without gibberellic acid may be necessary. Cytokinins may be needed to stimulate a dormant meristem to grow. The nutrient medium should be autoclaved at 105° C (20 psi) for 20 min. Heat labile substances must be sterilized by millipore filtration and added aseptically. As soon as meristem tips have developed into rooted plantlets (Fig. 3) they may be transferred to small pots containing a sieved compost mixture (Fig. 4). To maintain humidity the plantlets are covered with inverted glass beakers until they are well established. If it is not possible to induce root formation, grafting of the rootless plantlet onto healthy seedling may lead to an established plant (HÜTH and BODE, 1970). BUYS (1969), however, prefers carnation plantlets without roots to be transferred to soil. In his opinion the roots, growing at the expense of the developing meristem tip in the tube are of little use in the soil. When the plants are large enough they are tested for presence of virus, serologically, under the electron microscope or by inoculation onto indicator species. Since it usually takes time for the initially minute traces of virus—possibly present

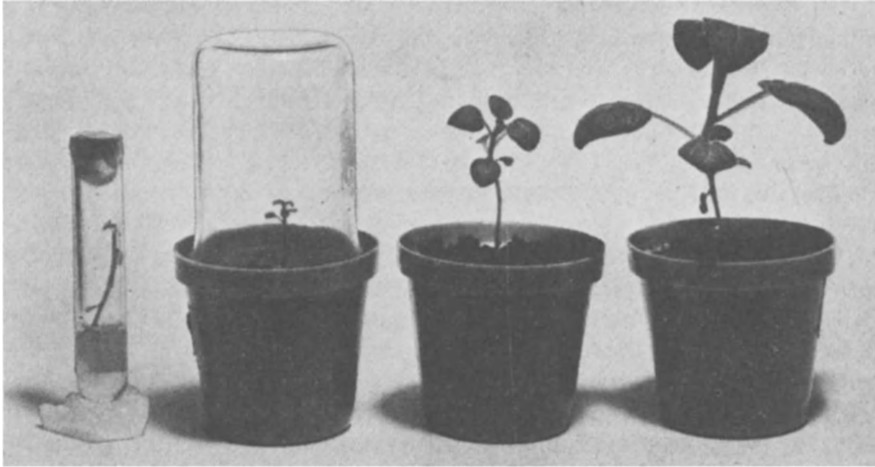

Fig. 4. Various stages showing the transfer of virus-free potato plants from test tubes to soil

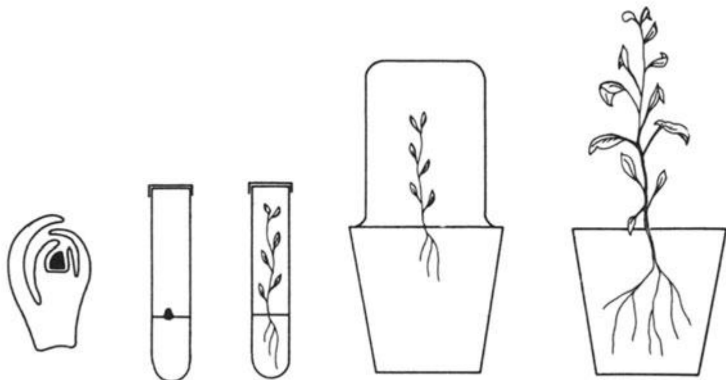

Fig. 5. Schematic representation for the isolation and culture of meristem tips, regeneration of virus-free plants, and their subsequent transfer to soil

in the explant—to build up to demonstrable concentration, repeated testing is essential before a clone may be declared free from the virus tested for. Various steps in the isolation and culture of meristem tip and their subsequent regeneration into virus-free plants is diagrammatically represented in Figure 5.

3.3 Factors Affecting Meristem Culture

There is no simple rule for the success of meristem-tip culture, since it depends partly on the nature of the viruses present: Some viruses are more readily eliminated than others. Potato plants obtained from meristem and one leaf primordium were found free from potato leaf-roll virus, 70–80% were free from potato virus A and potato virus Y, and not more than 50 out of 500 plants were free from potato virus X. Of many plants grown from meristems of "Eersteling", a symptomless

carrier of potato virus S and potato virus X, only two had lost virus X but were still infected with virus S (TEN HOUTEN *et al.*, 1968). Similarly, it was observed that carnation mottle virus was less readily eradicated from carnation plants than were ringspot, carnation vein mottle and latent viruses (STONE, 1968). Results of meristem-tip culture often vary between varieties. Within a year of excision potato virus S-free tubers of "Red Pontiac" were obtained from several meristems. On the same medium other potato varieties required two years to reach this stage, and two selections of "Kennebec" grew very poorly and only produced plants still containing virus S (TEN HOUTEN *et al.*, 1968). Similar results were obtained with carnations, strawberries (unpublished data) and chrysanthemum (HAKKAART and QUAK, 1964). Mostly meristem-tip culture is done using 0.6–0.8% agar as support. In most plant species the roots grow down into the agar to the bottom of the tube while in others like carnation, they often grow upward against the glass. In such cases liquid medium with folded ash-free filter paper bridges may be preferable (HELLER, 1949). STONE (1963) found this system superior to agar, giving better aeration, better root development and ease of removal of the plantlet. Of strawberry, gooseberry (VINE, 1968; WALKEY, 1968) and rhubarb meristem tips have been grown successfully on filter paper. In the experiments of PENNAZIO and REDOLFI (1973) potato plantlets cultivated on liquid substrate were more vigorous than those grown on agar media and had better developed root systems. Comparing agar and liquid media, MELLOR and STACE-SMITH (1969) found that for potato meristem tips some growth regulators are more detrimental to excised tips in liquid than in solid media. Probably growth regulators should be used at lower concentrations in liquid than is recommended for use in solidified media.

The pH of a medium may be a limiting factor for growth and in general should be between 5.5 and 5.8. A downward shift of the pH during autoclaving should be taken into account. STONE (1963) reports that carnation meristem tips grow considerably better on pH 5.5 (59%), than on pH 6 (4%). The buffering capacity of media may also vary. MELLOR and STACE-SMITH (1969) observed that the pH of the medium dropped within a week from 5.7 to 5.4. Rooting was inhibited on media with a low initial pH.

In most plants, in addition to the terminal bud, lateral axillary buds are available which may also be used for meristem-tip culture. According to HOLLINGS and STONE (1968) the apical meristem of chrysanthemum gives better survival: of some 5000 meristem tips isolated, 32% from the terminal buds grew into mature plants, compared with 18% from the lateral buds. In general, for reasons of efficiency, it is advisable also to dissect axillary buds. With bulbs and corms it is also feasible, though not always practicable.

Comparing growth of potato meristem tips derived from sprouts developed in light and darkness, respectively, HUTH and BODE (1970) found the latter unsatisfactory, presumably due to high phenol-oxidase activity. Plantlets rooting may also be affected by explant size. STONE (1963), who found carnation tips smaller than 0.2 mm unlikely to root, and those larger than 0.75 mm produced plants that still contained mottle virus. Tips between 0.2 and 0.5 mm had the best chances of producing virus-free plants. In cassava only explants exceeding 0.2 mm formed complete plants (KARTHA and GAMBORG, 1975). Those less than 0.2 mm in length produced either callus or callus with roots. Both in Indian and in Nigerian

cultivars of cassava the presence of leaf primordia was found not to be essential for plant regeneration. For rhubarb, however, it was necessary to dissect tips with 2–3 primordial leaves—smaller tips did not grow (WALKEY, 1968). In this instance virus retention was not related to tip size, which ranged from 0.4–2.4 mm.

Culture tubes containing meristem tips are placed about 30 cm beneath fluorescent light. For most plants 16 h illumination a day, and temperatures around 22° C are satisfactory. Potato meristem tips grow better at 25 °C (QUAK, unpublished), whereas bulbs may require temperatures between 12 and 15° C (HOLLINGS and STONE, 1968). For *Pelargonium* meristem tips PILLAI and HILDEBRANDT (1968) report that a definite dark period is necessary, possibly to minimize the inhibitory effect of polyphenolic substances.

Several workers have experienced seasonal influence on meristem tip culture processes. STONE (1963) observed better survival of carnation meristem tips in early spring and early autumn than in winter and summer. VAN OS (1964), however, states that carnation meristem-tip isolations made in winter rooted more easily, but that meristem tips isolated in summer produce a higher percentage of virus-free plants.

MELLOR and STACE-SMITH (1969) and QUAK (unpublished) found that for most potato varieties tips excised in spring and early summer rooted more readily than those taken later in the year. With bulbs and corms, the best results may be expected when they are dissected at the end of their dormancy period.

Equally or more important than the influence of the season may be the quality of the plant material. The regeneration of meristem is favorably affected by growing the plants under optimal conditions before use.

3.4 Meristem Culture after Heat Treatment

Even on media considered to be favorable for their growth, few meristems develop and very few plantlets may be virus-free. Therefore several workers have first heat treated the plant material and then cultured larger meristem tips from them.

The excised meristem tips are up to 1 mm long with two or three leaf primordia. It is assumed that during heat treatment the virus multiplication is inhibited. As heat treatment may impede the metabolism and reduce growth of the meristems, trials are needed to find out how long meristems can be kept at high temperatures and still develop in reasonable numbers, thus obtaining the maximum proportion alive and virus-free. Working with chrysanthemum cv. Blanche Portevine Suprême HAKKAART and QUAK (1964) found that material heat treated for 10, 20, or 30 days, respectively, produced percentages of virus-free plants increasing from 9 to approximetely 90. However, heat treatment for 40, 50, and 60 days, did not change this percentage, but markedly decreased the absolute number of meristems developing into plants. The apparent affect on the meristems varied with the varieties studied: under comparable conditions cv. Migoli produced very few plants after 20 and 30 days of heat treatment, which, however, were free from virus B for 100 and 70%, respectively, as compared with 9% of the non-heat treated material. HOLLINGS and STONE (1967) reported a high propor-

tion of healthy chrysanthemums recovered from leaf mottling viruses by meristem-tip culture and heat treatment. ASATANI (1972), however, found no correlation between the length of heat treatment and proportions of explants free from virus. Other factors were involved when 100% of explants were freed from virus with three cultivars and 98% with one cultivar by meristem-tip culture only.

Certain potato viruses may pose problems in elimination by meristem-tip culture which pre-heat treatment may help to overcome. MACDONALD (1973) freed the cultivars Duke of York and Royal Kidney from viruses X and S by culturing meristem tips isolated from sprouts of heat-treated tubers. Cultivar Doon Star Wilding was freed from virus X but not from virus S. STACE-SMITH and MELLOR (1968) observed that the size of the potato meristem tip was of critical importance in virus eradication from buds taken after short or no heat treatment, but was of less importance as the duration of heat treatment lengthened. The number of explants from cultivar White Rose free from virus X increased progressively with treatment until it reached 50% after eight weeks and nearly 100% after 18 weeks. They found it more difficult to eliminate virus S than virus X. Comparable results were reported by ZAKLUKIEWICZ (1971), who isolated meristem tips from axillary buds of potato plants of several varieties, previously subjected to temperatures of 33–37° C for 4–12 weeks. Only 87 plants (about 4% of the total number of isolates) were obtained, out of which 81 were virus-free. PENNAZIO (1971), working with Italian potato cultivars, also experienced the favorable effect of combining thermotherapy with meristem-tip culture to eradicate potato viruses.

Tips excised by VINE (1968) from strawberry plants, after a week or more at 35° C, grew rapidly and a higher proportion reached maturity, than those grown from untreated plants. They were also largely free from crinkle and vein chlorosis. Similarly MULLIN *et al.* (1975), eliminating pallidosis from strawberry, found meristems growing faster when source plants were heat treated and more of them developing into plants, than those taken from non-heated sources. Heat treatment followed by shoot-tip culture has also successfully eliminated certain virus symptoms from cultivars of *Ipomoea batatas* (OVER DE LINDEN and ELLIOTT, 1971).

Usually heat treatment is applied to the source material prior to excision of the meristem tips, WALKEY and COOPER (1975), however, subjected infected meristem tips of *Nicotiana rustica*, grown in static or shake culture, to 32° C. By this method cucumber mosaic virus and alfalfa mosaic virus were eradicated or their concentrations greatly diminished, whereas similar cultures kept at 22° C remained infected. Tobacco mosaic virus was not eradicated from shake cultures at 32° C and its concentration was sometimes higher than at 22° C.

It may be concluded that in those instances where viruses are difficult to eradicate by meristem-tip culture, the application of heat treatment prior to excision of the explants should be considered. This combined procedure may also be advantageous when, as with carnation, only small explants, developing slowly and in a low proportion produce virus-free plants. In such instances pre-heat treatment allows the excision of 1–2 mm large tips, rather than 0.1–0.3 mm meristems, with better chances of growth and still leading to virus-free plants (QUAK, 1957; VAN OS, 1964). For a list of plants in which viruses have been eliminated see Table 2.

Table 2. Species in which complete plants have been obtained by the culture of meristem tips, and viruses eliminated

Plant species	Virus eliminated	Reference
Allium sativum (garlic)	mosaic virus	MORI (1971)
		QUIOT *et al.* (1972)
	mosaic virus, onion yellow dwarf mosaic virus, unidentified virus	HAVRÁNEK (1973)
Ananas sativus (pineapple)	[a]	SITA *et al.* (1974)
Brassica oleracea (cauliflower)	turnip mosaic virus cauliflower mosaic virus	WALKEY *et al.* (1974)
Brassica oleracea var. gemmifera (brussels sprouts)	[a]	CLARE and COLLIN (1973)
Caladium hortulanum	dasheen mosaic virus	HARTMAN (1974)
Colocasia esculenta (taro)	dasheen mosaic virus	HARTMAN (1974)
Chrysanthemum sps.	virus B	HAKKAART and QUAK (1964) QUAK and HAKKAART (1966)
	stunt	HOLLINGS and STONE (1970)
	tomato aspermy virus virus B, vein mottle virus	ASATANI (1972)
	tomato aspermy virus, virus B	PALUDAN (1973)
	chlorotic mottle virus, stunt	PALUDAN (1974)
Cymbidium sps.	cymbidium mosaic	MOREL (1960)
Dahlia sps.	dahlia mosaic virus	MOREL and MARTIN (1952)
	complex of viruses	MORI (1971)
Dianthus barbatus	ringspot virus, mottle virus, vein mottle virus, latent virus	STONE (1968)
Dianthus caryophyllus (carnation)	ringspot virus, mottle virus, vein mottle virus, latent virus	QUAK (1962); VAN OS (1964) STONE (1968)
	streak virus	PHILIPS (1962)
	mottle virus	STONE (1963)
	mottle virus, unidentified virus	MAIA *et al.* (1969)
	etched ring virus	PALUDAN (1970)
	[a]	PENNAZIO (1973)
	mottle virus	KOWALSKA (1974)
	vein mottle virus	PALUDAN and BEGTRUP (1974)
Fragaria sps.	vein banding, crinkle, yellows virus complex	MILLER and BELKENGREN (1963)
	complex of viruses	QUAK (1964)
	latent C virus	MCGREW (1965)
	yellow edge (virus 2), vein chlorosis (virus 4), crinkle (virus 3), latent A virus	VINE (1968)
	complex of viruses	MORI (1971)
	[a]	ADAMS (1972)
	pallidosis virus, mild yellow edge virus, mottle virus	MULLIN *et al.* (1974)
	mottle virus, edge virus, crinkle virus	KACHARMOZOV and IZVORSKA (1974)
Freesia sps.	freesia mosaic virus, phaseolus virus 2	BRANTS and VERMEULEN (1965) BRANTS (1968)

Table 2 (continued)

Plant species	Virus eliminated	Reference
Gladiolus sps.		ZIV *et al.* (1970)
	unidentified viruses	SIMONSEN and HILDEBRANDT (1971)
Hippeastrum sps. (amaryllus)	mosaic virus	NOWICKI and O'ROURKE (1974)
Humulus lupulus (hop)	prunus necrotic ringspot virus, hop latent virus	VINE and JONES (1969) GIPPERT *et al.* (1974) ADAMS (1975)
Hyacinthus sps.	hyacinth mosaic virus	VAN SLOGTEREN (1966)
	hyacinth mosaic virus, lily symptomless virus	ASJES *et al.* (1974)
Ipomoea batatas (sweet potato)	internal cork virus, rugose mosaic virus, feathery mottle virus	MORI (1971)
	unidentified viruses	OVER DE LINDEN and ELLIOT (1971); ALCONERO *et al.* (1975)
Iris sps.	iris mosaic virus	BARUCH and QUAK (1966)
	unidentified virus	MORI (1971)
Lilium sps. (lily)	unidentified viruses	NISHIZAWA and NISHI (1966)
	[a]	SHERIDAN (1968)
	cucumber mosaic virus, lily mosaic	MORI (1971)
	unidentified viruses	ALLEN (1974)
	hyacinth mosaic virus, lily symptomless virus	ASJES *et al.* (1974)
Malus (apple)	[a]	ELLIOTT (1972), WALKEY (1972), QUOIRIN (1974)
Manihot utilissima (cassava)	unidentified viruses	KARTHA *et al.* (1974b)
	mosaic disease	KARTHA and GAMBORG (1975)
Musa sps. (banana)	cucumber mosaic virus, unidentified virus	BERG and BUSTAMANTE (1974)
Narcissus tazetta	arabis mosaic virus, narcissus degeneration virus	STONE (1973)
Nerine sps.	nerine latent virus, unidentified virus	HAKKAART *et al.* (1975)
Pelargonium sps. (geranium)	tomato ringspot virus unidentified virus	PILLAI and HILDEBRANDT (1968)
	cucumber mosaic virus, tomato black ring virus, tomato ringspot virus	GIPPERT and SCHMELZER (1973)
	[a]	BEAUCHESNE (1974)
Petunia sps.	tobacco mosaic virus	MORI (1971)
Phaseolus multiflorus	[a]	GRIFFAUT (1971)
Pisum sativum (pea)	[a]	KARTHA *et al.* (1974)
Prunus sps.	[a]	BOXUS and QUOIRIN (1974)
Ranunculus asiaticus	unidentified viruses	MAIA *et al.* (1973)
Rheum rhaponticum (rhubarb)	turnip mosaic virus, cherry leaf-roll virus, strawberry latent ringspot virus, cucumber mosaic virus	WALKEY (1968)

Table 2 (continued)

Plant species	Virus eliminated	Reference
Ribes grossularia cv. uva-crispa (gooseberry)	vein banding	JONES and VINE (1968)
Rubus ideaus (raspberry)	mosaic	PUTZ (1971)
Saccharum officinarum (sugarcane)	mosaic	MORI (1971), LEU (1972)
Solanum tuberosum (potato)	paracrinkle virus, virus X	KASSANIS (1957)
	virus X, virus S	QUAK (1961), YORA and TSUCHIZAKI (1962)
		MOREL and MULLER (1964)
	virus X, virus Y, virus S	SVOBODOVA (1964)
	virus A, virus X, virus S	KASSANIS and VARMA (1967)
	virus A, virus X, virus Y, virus S, virus M	MOREL *et al.* (1968)
	virus X, virus S	STACE-SMITH and MELLOR (1968)
	virus S	HUTH and BODE (1970)
	spindle tuber	STACE-SMITH and MELLOR (1970)
	virus X, virus Y, virus S, leaf-roll virus	MORI (1971)
	virus A, virus S	ŠIP (1972)
	virus X, virus S	MACDONALD (1973)
	virus S, virus M	PETT (1974)
Vitis rupestris (vine)		GALZY (1972)
Xanthosoma brasiliense (coçoyam)	unidentified virus	HARTMAN (1974), STARITSKY (1974)

[a] Plants obtained were not tested for virus presence.

3.5 Virus in Meristematic Tissue

Meristem-tip culture has intrigued plant pathologists for its theoretical interest as well as for its practical importance. If the nature of the phenomena involved were understood, some of the difficulties encountered would be easier to overcome.

In the fifties, it was surmised from the virus-inhibiting effect of certain growth hormones, that a high concentration of hormones in meristems inactivated the invading virus. This hypothesis has never been proved. It has been concluded by WU *et al.* (1960) that the concentration of virus was diminished by rapid growth of callus. Infection experiments with suspensions of tobacco callus cells suggested a competition between cell division and virus multiplication. In actively dividing tissue, synthesis of normal nucleoproteins would prevail and later, during cell elongation there was synthesis of viral nucleoprotein (QUAK, 1965). Observations by CROWLEY and HANSON (1960) support this hypothesis. They correlated the length of the virus-free tip of a tobacco mosaic virus-infected tomato root with the zone where

mitosis occurred. A growth inhibitor in the medium caused shortening of the mitotic zone in the root tip. The virus-free zone diminished to the same extent. In the same period, however, virus particles were detected in meristematic tissue. According to HOLLINGS and STONE (1964) meristem tips excised from carnation plants infected with carnation mottle virus contained this virus in such a concentration that they could demonstrate it with the local lesion host *Chenopodium amaranticolor*. However, after 30 h on nutrient medium, the meristems were no longer infectious, suggesting that contact with the nutrient medium eliminated virus present in the apex. Similar results were obtained by WALKEY and WEBB (1968) and WALKEY *et al.* (1969) in meristem tips of *Nicotiana rustica* infected with cherry leaf-roll virus or arabis mosaic virus. They excised meristems, without leaf primordia, and homogenized each one in a drop of potassium phosphotungstate, which enhances the visibility of isometric virus particles. In the electron microscope the homogenate clearly showed virus particles though most cultures of such tips produced healthy plants. Electron micrographs have also provided evidence of the presence of tobacco ringspot virus in apical initials of bean root meristems (CROWLEY *et al.*, 1969) and in apical meristems of tobacco shoots (ROBERTS *et al.*, 1970). APPIANO and PENNAZIO (1972), KRYLOVA *et al.* (1973), and ZAKLUKIEWICZ (personal communication) have demonstrated the presence of the elongate viruses X, M, and S in excised meristem tips of potato, which may well produce plants free from these viruses.

At present no general explanation is available for the fact that some viruses are and other are not eliminated in meristem-tip culture; both normal cell metabolism, and virus replication being intimately involved. Differences in the ease with which viruses can be eradicated could be due to differences in inactivation on the nutrient medium as well as to differences in their distribution in the apex. The composition of the culture medium might well be of importance in inactivating virus from the meristem tips and also determining the success of their culture.

The suggested possibility of nucleoprotein synthesis required for cell division and virus synthesis being competitive does not seem to have general validity, but might account for the favorable effect of heat treatment on elimination of viruses in apices (WALKEY and COOPER, 1972). Despite the theoretical basis not being fully understood, it does not detract from the practical importance of meristem-tip culture for recovery of clones fully virus-infected.

3.6 Effect of Elimination of Viruses

Meristem-tip culture has been used by many workers to obtain virus-free clones of vegetatively propagated plants. The aim of application of the technique may be twofold. The obtained plants may provide material with which the effects of viruses can be studied, and it may form the basis for the commercial growing of the clones involved. However, it should be kept in mind that, since the elimination of virus has not brought about immunity, reinfection must be expected. The speed of reinfection and the nature of preventive measures to be taken, are largely determined by the epidemiology of the viruses involved.

Although various viruses of carnation were known, it was not before 1964 that HAKKAART could describe symptoms and assess losses caused by carnation mottle, ringspot vein mottle and latent viruses in the carnation cv. William Sim. Although no clear leaf symptoms were obtained by artificial infection with mottle virus, the growth of infected plants was markedly reduced as was later confirmed by PALUDAN (1968). Ringspot and vein mottle caused leaf symptoms and depressed both quality and quantity of the yield, ringspot-infected plants being more prone to production of flowers with split calyx than virus-free plants. Vein mottle virus caused flower color breaking.

It is not usual that "new" viruses are detected in "virus-free" material obtained through meristem-tip culture. Since testing of this material is necessarily limited to known viruses others may remain, as was predicted by HAKKAART (1964) for carnation etched ring virus. Obviously the testing procedure had to be extended to include a test for this latter virus. STONE (1973) eliminated arabis mosaic virus and narcissus degeneration virus from *Narcissus tazetta*, cv. Grand Soleil d'Or by meristem-tip culture. The virus-free bulbs grew rapidly and showed greater vigor than the ordinary stock. The flowers were larger, richer in color and more per stem than those obtained from infected bulbs.

WALKEY and COOPER (1972) found commercial cultivars of rhubarb in Britain widely infected with viruses. They could not determine the effect of virus infection on the vigor and yield of these cultivars until virus-free stock became available as a result of meristem-tip culture (WALKEY, 1968). A 60–90% increase in yield of petioles was observed as compared with virus-infected plants.

The production of cauliflower seed is from specially selected and clonal-maintained mother plants, which as a result of being propagated vegetatively for many years, may become heavily infected with viruses. This may lead to reduced seed yield and death of plants. Using tissue culture of cauliflower WALKEY *et al.* (1974) succeeded in regenerating vigorous virus-free plants in some clones.

From a number of cultivars of *Pelargonium zonale* GIPPERT and SCHMELZER (1973) eliminated viruses by meristem-tip culture. Even though viruses do not always cause symptoms in *Pelargonium* these authors observed that plants cultured from meristem tips were more vigorous than untreated plants and produced 20–30% more cuttings. In addition the rooting capacity of these cuttings showed improvement, so that the total production increased by about 35%.

Various cultivars of chrysanthemum, freed from chrysanthemum virus B, have shown a considerable increase in growth, and give, both quantitatively and qualitatively, better yields (HAKKAART, unpubl.). Numerous publications (see Chap. V.3. of this Volume) have been devoted to losses in potato growing caused by virus infection. For the production of seed potatoes of high quality, tubers used for planting should obviously be free from virus infection. Tubers used for growing potatoes for consumption, or for industrial processing need not be completely free from virus. However, from the literature it may be concluded that viruses, alone or in combination with one another, seriously affect plant growth and losses of up to 95% for viruses like potato virus Y and potato leaf-roll virus have been observed depending on the variety involved, even potato viruses M, S, and X which are generally considered to be less severe may cause losses of up to 70%. So both in production of seed potatoes and of ware-potatoes the recovery of economically

important, but fully virus-infected varieties should be considered. When a variety is fully infected with a latent virus, its elimination, if leading to increased yield may also beneficially remove a source of infection for other cultivars, as was the case with "Eersteling", a symptomless carrier of the potato viruses X and S (QUAK, unpubl.). The Italian cv. San Michele, freed from unknown viruses by meristem-tip culture in a field trial showed 60% more yield (GREGORINI and LORENZI, 1974).

In England a virus-free stock of cv. King Edward, previously fully infected with paracrinkle, a strain of potato virus S, was propagated in seed-growing areas and has been used for yield experiments. The new virus-free stock was compared with seven of the best commercial stocks in nine centers of the country for four consecutive years. The average yield of the stock was 10% more than that of the commercial stocks and the tubers were more uniform in size (KASSANIS and SCHWABE, 1961).

It is not unknown that cultivars freed from one or more viruses, even if they are latent, have a more or less changed appearance: for instance, virus-free plants of cv, Eersteling are darker green and lusher than those latently infected with potato viruses X and S [General Netherlands Inspection Service (NAK) Wageningen, unpublished]. It is generally believed that meri-clones with an increased mutation rate, particularly when the culture of the meristem tips includes callus growth, lead to genetic aberrations although no statistical data on mutation frequency are available. A prerequisite, however, to optimal benefit of nuclear stock material of meri-clones is continued selection for quality and type. In this respect the meri-clone is not different from other clones. The culture of meristem tips presents the one means of vegetative propagation in which viruses are not advanced. The genetical composition of the material remains generally unaltered. This means that a virus-free clone of high quality, can only be expected if meristem-tip culture (or heat treatment) is applied to high quality, but virus-infected material. A clone after therapeutic treatment would still have its negative properties, although it will be virus-free.

In freeing economically important cultivars from vegetatively propagated crops it is not enough to eliminate the effects of viruses. The cultivars involved keep their susceptibility to virus infection, and from the beginning, measures should be aimed at prevention of their massive re-infection. These measures are determined on the one hand by the epidemiology of the viruses, which may more or less be readily transmitted mechanically or by vectors, and on the other hand by the crop involved, which may be glasshouse or field grown and have a one or several years' culture period.

In some cases, as with fruit trees, re-infection with most viruses is not probable if both rootstock and scion are virus-free, since the majority of the viruses involved have no vectors. In crops, such as carnation, chrysanthemum, strawberry and potato, re-infection with insect- or mechanically transmitted viruses is a constant threat, which can be diminished by a variety of sanitary measures. In any case, nuclear stock plants must frequently and individually be tested for presence of virus, so that re-infection of this first grade material can be checked in time. Second grade material, available to growers, is usually tested randomly. To qual-

ify for a certain certificate, a previously set norm for virus infection should not be exceeded.

Obviously re-infection is fully excluded as long as virus-free plantlets, developing from excised meristem tips, remain in their tubes. Therefore, it has been suggested (BOXUS, 1974) for strawberry to maintain nuclear stock material in vitro. This implies that, in order to reach the numbers of plants required, rapid propagation should take place in vitro (see Chap. I.7 of this Vol.). If this includes a callus stage, the increased mutation rate should not be ignored.

References see page 636.

3. Virus-Free Potatoes by Tissue Culture

F. C. MELLOR and R. STACE-SMITH

1. Introduction

1.1 Historical

The potato, *Solanum tuberosum* L., is one of the world's most important food crops, especially in the temperate regions of the Northern Hemisphere. In 1933 more than 600 cultivars were listed (BURTON, 1966) and the number increases each year. Many cultivars, which were once popular and productive, gradually decreased in vigour and cropping capacity before it was recognized that the degeneration was largely due to infection with one or more viruses. When these caused obvious foliar symptoms the infected plants could be rogued out, but symptomless viruses were more difficult to detect and control.

As knowledge of potato viruses advanced, and techniques for virus detection improved, it became evident that virtually all of the important cultivars were infected with one or more of the mild or latent viruses. In selecting foundation stock for use in certification schemes, the presence of these latent viruses was recognized but accepted, since no virus-free clones were available. It is only in recent years, using the techniques discussed here (see Chap. V.2 of this Vol.), that truly virus-free stock has become available to the potato industry.

1.2 Disease Losses

When virus-free potatoes were first obtained, tests were made to estimate yield losses attributed to the individual viruses. The results were variable, since cultivars differ in their sensitivity to a particular virus, and the viruses exist as strains of varying severity. The early work on the effect on yield of potato virus X (PVX) was reviewed by Norris (1953). He noted that PVX infection may cause yield losses between 5 and 75% according to the strain of the virus, the cultivar, and other factors, one of which would be the presence of other latent viruses in addition to PVX. Potato virus S (PVS) was one of these which remained undetected until 1951 when it was discovered in the Netherlands. It was found to be widespread wherever potatoes were grown, and would undoubtedly be present, unrecognized, in many of the cultivars used in the earlier yield trials. Other viruses that may influence yield include potato virus A (PVA) and potato virus Y (PVY).

Following the use of a meristem culture technique (see Chap. V.2 of this Volume) to produce a virus-free plant of King Edward (KASSANIS, 1957), the clone was propagated and compared with several other clones which were infected with

paracrinkle virus, now known as potato virus M (PVM). The virus-free clone produced more vigorous haulms and about 10% higher yield, attributed to more tubers rather than larger ones (BAWDEN and KASSANIS, 1965).

More accurate estimates of yield loss have recently been made by introducing known viruses into virus-free plants, and comparing the infected plants with virus-free plants of the same clone. WRIGHT (1970) investigated the combined effects of PVX and PVS on the yield of Netted Gem and White Rose, two commonly grown cultivars in North America. In three out of four locations, yields from the virus-free clones were significantly higher (11–38%) than those from comparable infected material. In a study of the combined effect of PVX and PVA, GREGORINI and LORENZI (1974) found yield increases up to 60%. BEEMSTER and ROZENDAAL (1972) observed that certain necrosis-evoking strains of PVX caused more than 50% loss in some cultivars; that PVS losses were 10–15%; and that combined infections of PVA and PVY caused serious losses.

The beneficial effects of virus eradication may be partly offset by increased susceptibility to invasion by parasitic fungi. MÜLLER and MUNRO (1951) demonstrated that the foliage of potato free from PVX and PVY was highly susceptible to *Phytophthora infestans* (Mont.) de Bary. JONES and MULLEN (1974) found that PVX-free tubers, when left in the ground for 2–3 weeks after top-kill, were more susceptible to *Fusarium* dry root than were camparable PVX-infected tubers, although tubers harvested 4–5 weeks after top-kill showed no differences in susceptibility. These observations suggest that virus infection alters the nutritional or physiological status of the potato plant.

The possible increase in susceptibility to fungal infection in virus-free potatoes is unlikely to detract greatly from the value of a virus-free program. The benefits more than compensate for any adverse effects. However, in some regions cultural modifications may be needed to counteract increased susceptibility to other pathogens.

In summary, there is overwhelming evidence that, although PVX and PVS are usually symptomless in potato, they cause significant decreases in yield. With the advances that have been made in meristem tip culture, even the most stable strains of these two viruses can be eradicated, and virus-free clones developed in almost all the important cultivars. Furthermore, clones arising from meristem culture are also free from pathogenic bacteria and fungi. With scrupulous attention to sanitation during increase, top quality material can be available to any potato certification scheme.

1.3 Methods of Eradication

Tissue culture of potato buds as a means of developing virus-free plants from infected stock has developed to a point where success is assured. Three methods have been used in attempts to isolate virus-free tissue that will regenerate into a rooted plant. The first is based on observations that virus concentration diminished from older to younger vegetative parts of the infected plant. By isolating the

apical meristem (MOREL and MARTIN, 1955) or the root tips (BAJAJ and DIONNE, 1966) from infected plants and growing them on a nutrient medium, virus-free tissue cultures have been established; but these do not always develop into rooted plants. The second is the use of antimetabolites (NORRIS, 1954), either applied to the infected plant before bud excision, or incorporated into the nutrient medium. Some antimetabolites decrease the concentration of virus without irreparable harm to the plant tissue. The third is to subject the infected plant to temperatures near 37° C (KASSANIS, 1950), which retards or inhibits the multiplication of some viruses. Meristematic tips excised from heat-treated plants are often free from viruses that are not easily eliminated by meristem culture alone.

2. Meristem Culture

2.1 Tissues for Culturing

Meristematic buds from apices of stems or tuber sprouts, or from leaf axils, are usually selected for tissue culture. Their advantage is that the incipient shoot has already differentiated; to establish an independent plant, only elongation and root differentiation are required. It may be of little practical value to culture other tissue, but it should be recognized that almost any part of the potato plant has the potential of producing an independent plant. Non-meristematic tissue must undergo a developmental process which usually involves the formation of callus, and its differentiation into embryos and plantlets.

BAJAJ and DIONNE (1966) investigated the possibility of obtaining PVX-free plants by culturing potato roots. They developed three root cultures which were free from PVX and they later (1968) succeeded in producing nodule-like structures on cultured roots. These structures tended to turn green but they did not develop into shoots.

Tuber tissue has not been used as a source of virus-free clones, although the growth and differentiation of parenchymatous tuber tissue has been studied. OKAZAWA *et al.* (1967) found that auxin was indispensable for initiating callus formation, and kinetin for its continuous growth and proliferation. When discs of tuber tissue 16 × 10 mm were cultured, tiny sprouts developed on the surface of the discs. ANSTIS and NORTHCOTE (1973) subcultured pieces of callus from tuber tissue but no shoots were formed, even though roots developed on one callus.

Potato anther culture may also have possibilities. Recently, DUNWELL and SUNDERLAND (1973) have reported the induction of androgenesis and the regeneration of haploid plants.

Up to now, most experiments with stems, tubers, roots, and anthers of potatoes have been to investigate growth phenomena in callus rather than virus eradication, which requires differentiation into rooted shoots to be of practical value. The many observations on tissue differentiation suggest that, although the potential is present, there are numerous unsolved problems. There appears to be little value at present in using tissue other than meristem for virus eradication.

2.2 Culture Media

Increased concentrations of macroelements in the nutrient medium led to greater success in development of excised meristems into rooted plantlets. The minerals in each of several solutions that have been used for potato are listed in Table 1. MOREL and MULLER (1964) improved the medium devised by MOREL and MARTIN (1955) by greatly increasing the amount of potassium and adding ammonium sulfate and gibberellic acid (GA_3). MURASHIGE and SKOOG (1962) developed a medium (MS-62) which was even richer in ammonium and potassium, and contained nearly four times as much nitrate. Comparisons of several media were made by STACE-SMITH and MELLOR (1968a), HUTH and BODE (1970), CHRISTENSEN (1970), and TAPIO (1972) and they all found MS-62 to be superior. The increasing proportion of plantlets that developed with greater concentration of nutrients is shown in Table 2.

Table 1. Mineral nutrients (mg/l) that have been used in several media for meristem tip culture of potato

	WHITE, 1954 (MANZER, 1958)[a]	HELLER, 1953 (GAUTHERET, 1959)	MOREL and MARTIN, 1955 (PALUDAN, 1971)	MOREL and MULLER, 1964	MURASHIGE and SKOOG, 1962
Macronutrients:					
$Ca(NO_3)_2 \cdot 4H_2O$	288		500	500	
KNO_3	80		125		1900
$NaNO_3$		600			
NH_4NO_3					1650
$(NH_4)_2 \cdot SO_4$				1000	
KCl	65	750		1000	
KH_2PO_4			125	125	170
$NaH_2PO_4 \cdot 4H_2O$	19	125			
$CaCl_2 \cdot 2H_2O$		75			440
$MgSO_4 \cdot 7H_2O$	737	250	125	125	370
Na_2SO_4	200				
Micronutrients:					
$FeCl_3 \cdot 6H_2O$		1		1	
$Fe_2(SO_4)_3$	2.5		25		
$FeSO_4 \cdot 7H_2O$					27.8
Na_2-EDTA					37.3
$MnSO_4 \cdot 4H_2O$	6.7	0.1	0.8	0.1	22.3
H_3BO_3	1.5	1	0.025	1	6.2
$ZnSO_4 \cdot 4H_2O$	2.2	0.8	0.04	0.8	8.6
KI	0.75	0.01	0.25	0.01	0.83
$Na_2MoO_4 \cdot 2H_2O$					0.25
$CuSO_4 \cdot 5H_2O$		0.03	0.025	0.03	0.025
$AlCl_3$		0.03		0.03	
$NiCl_2 \cdot 6H_2O$		0.03	0.025	0.03	
$CoCl_2 \cdot 6H_2O$			0.025		0.025

[a] Where formula given is not taken from the original source the reference used is shown in parentheses.

Table 2. Influence of nutrient medium on development of potato meristems in culture

Medium	Plantlets developed		Reference
	no.	%	
MM-55[a]	4/250[b]	1.6	KASSANIS, 1957
MM-55	5/94	5	YORA and TSUCHIZAKI, 1962
MM-55	18/430	4	ACCATINO, 1966
MM-64	20/193	10	KASSANIS and VARMA, 1967
MM-64	12/137	9	MACDONALD, 1973
MM-64	19/132	14	GREGORINI and LORENZI, 1974
MS-62	135/303	45	PENNAZIO, 1971
MS-62	30/45	67	PENNAZIO and REDOLFI, 1973

[a] References to media formulations: MM-55, MOREL and MARTIN, 1955; MM-64, MOREL and MULLER, 1964; MS-62, MURASHIGE and SKOOG, 1962.
[b] Number of plantlets developed per number of buds cultured.

Table 3. Organic supplements (mg/l) in media used for potato meristem tip culture

	WHITE, 1954 (MANZER, 1958)[a]	MOREL and MARTIN, 1955 (PALUDAN, 1971)	MURASHIGE and SKOOG, 1962
Biotin		0.001	
Ca-pantothenate		0.001	
Cysteine		0.01	
Glycine	3.0		2.0
Indol-3-acetic acid			1–30
Inositol		0.001	100
Kinetin			0.04–10
Napthaleneacetic acid		0.001	
Nicotinic acid	0.5		0.5
Pyridoxine · HCl	0.1		0.5
Thiamine · HCl	0.1	0.001	0.1
Glucose		40000	
Sucrose	20000		30000

[a] Where formula given is not taken from the original source the reference used is shown in parentheses.

The microelements most commonly used for nutrient culture are also listed in Table 1. Some investigators combine the macroelements of one medium with the microelements of another. Iron is usually supplied in the form of Fe-ethylenediaminetetraacetic acid (Fe-EDTA) rather than ferric chloride or sulfate. MURASHIGE and SKOOG (1962) pointed out that the chelating agent, EDTA, greatly improves the availability of iron, and possibly other micronutrients as well.

The organic supplements in some of the media for culture of potato buds are shown in Table 3. Investigation of the organic requirements for tissue culture is exceedingly complex. Some are interdependent: the need for one increases with the supply of another. The need for others may depend on available nutrients in the mineral solution, or on the type and age of tissue isolated. There has been

more study of the effect of organic supplements on callus and isolated cell cultures than on the development of meristems but some of these results may apply to the development of plants from meristems. MS-62 was developed for tobacco callus but it also proved excellent for culture of potato meristems.

In addition to the organic ingredients listed in Table 3, most investigators use GA_3. MELLOR and STACE-SMITH (1969) found that GA_3 had no appreciable effect on growth, and omitted it in their later work. From some cultivars nearly all the buds they excised developed into plantlets. From others, however, very few plantlets developed, and GA_3 might have improved the growth of these. PENNAZIO and REDOLFI (1973) found that rooting of meristems on liquid MS-62 without GA_3 was 17%, and with GA_3 it increased to 66%. They suggested the discrepancy between their results and those of MELLOR and STACE-SMITH might be due to different light regimes, cultivars, time of year, age of donor plant, or purity of chemicals. Another possible reason could be our routine transfer of developing buds to fresh medium when they show signs of stress.

We find that adding activated charcoal to MS-62 hastens development of excised buds and increases the number that develop into rooted plants. It is particularly useful for cultivars that do not root readily on MS-62. Beneficial

Table 4. Preparation of stock solutions[a] for culture medium[b] recommended for potato buds

Stock solution	Constituents	Conc. gm/l	Stock solution	Constituents	Conc. gm/l
A	NH_4NO_3	82.5	E	$MgSO_4 \cdot 7H_2O$	74.0
				$MnSO_4 \cdot 4H_2O$	4.46
B	KNO_3	95.0		$ZnSO_4 \cdot 7H_2O$	1.72
				$CuSO_4 \cdot 5H_2O$	0.005
C	H_3BO_3	1.24			
	KH_2PO_4	34.00	F[a]	$Na_2 \cdot EDTA$	7.45
	KI	0.166		$FeSO_4 \cdot 7H_2O$	5.57
	$Na_2MoO_4 \cdot 2H_2O$	0.05			
	$CoCl_2 \cdot 6H_2O$	0.005	G	Thiamine · HCl	0.02
				Nicotinic acid	0.1
D	$CaCl_2 \cdot 2H_2O$	88.0		Pyridoxine · HCl	0.1
				Glycine	0.4
			H[a]	Kinetin	0.4

[a] To prepare solution F: dissolve each constituent in 200 ml distilled water; heat $Na_2 \cdot EDTA$ solution; with continuous stirring add $FeSO_4 \cdot 7H_2O$ solution; when cool dilute to 1000 ml.
To prepare solution H: dissolve 4.0 mg kinetin in 10 ml 2% NaOH.
Stock solutions may be stored, refrigerated, up to a year with no appreciable deterioration.

[b] Preparation of final medium: To about 800 ml distilled water add 20 ml each of stocks A and B, 5 ml each of stocks C, D, E, F, and G, 0.1 ml of stock H, 30 gm sucrose, 100 mg inositol, 0.5 mg indol-3-acetic acid, and 0.1 mg gibberellic acid.
Dilute to 1000 ml and adjust pH to 5.7 with 2% NaOH.
Optional: add 10 mg activated charcoal per tube before dispensing medium.
Dispense medium into 12 × 100 ml test tubes, 3.5 ml per tube; autoclave 10 min at 12 lb pressure.

effect of charcoal has also been reported for tobacco anther culture (ANAGNOSTAKIS, 1974; BAJAJ *et al.*, 1975).

The culture medium may be solidified with 0.5–0.8% agar. For liquid culture the bud is usually supported on a filter paper bridge which also acts as a wick for the nutrient solution. The simplest bridge for small test tubes is a narrow filter paper strip with a single fold to form an inverted V with the point above the surface of the liquid. Development on filter paper is equal to, or better than, on agar.

The most convenient containers for meristem culture are test tubes, 12 x 100 mm, with heat-resistant caps which are designed to permit air exchange in the tubes. For liquid culture, the bud may be supported by a filter paper bridge but this is usually unnecessary. MELLOR and STACE-SMITH (1969) and PENNAZIO and REDOLFI (1973) reported that excised buds, and most developing plantlets, float on the surface of the liquid (Fig. 1, A, B, C). VASIL and HILDEBRANDT (1966) described a convenient method of preparing stock solutions for MS-62. Our adaptation of their method is given in Table 4.

2.3 Factors in Development and Rooting

2.3.1 Size of Excised Bud

The size of excised meristem influences rooting but the presence of leaf primordia, rather than the volume of tissue, appears to determine development. KASSANIS and VARMA (1967), using meristems 0.1 mm long found that the results were much better when a leaf primordium was included. STACE-SMITH and MELLOR (1968a) reported that buds, 1 mm or more long, rooted more readily than smaller buds. However, bud size also has a marked influence on elimination of viruses, particularly PVS. We usually reject buds less than 0.3 mm long because they are unlikely to root, and now we seldom use buds more than 0.7 mm long because they are liable to be infected. Within this narrow size range, there is little difference in rooting.

2.3.2 Cultural Environment

Environmental conditions usually used for potato meristems in culture are 20–23° C, with fluorescent light of 2000–4000 lux for a 16-h day. PENNAZIO and REDOLFI (1973) studied the effect of three light regimes: lamps which were rich in red light; lamps which approximated daylight; and a 2:1 mixture of both. Rooting under each regime was 18, 34, and 67% respectively. Moreover, the plantlets under mixed light were the most vigorous. They found no difference in percentage rooting between light intensity of 2000 and 4000 lux, but leaf and root development were better at 4000 lux.

We have compared the effect of temperature on growth of meristems from several cultivars by subjecting comparable buds on liquid MS-62 to a 16-h day at three temperatures. At 29° C, some cultivars grow quickly and rooted plantlets develop within 5 weeks. Development of others is abnormal: leaves are often larger, thicker, and paler than normal, and roots are sparse or absent. If these buds are not transferred to fresh medium they often die. At 26° C, growth is slower, but again shoot growth usually precedes root growth. Rootless shoots on

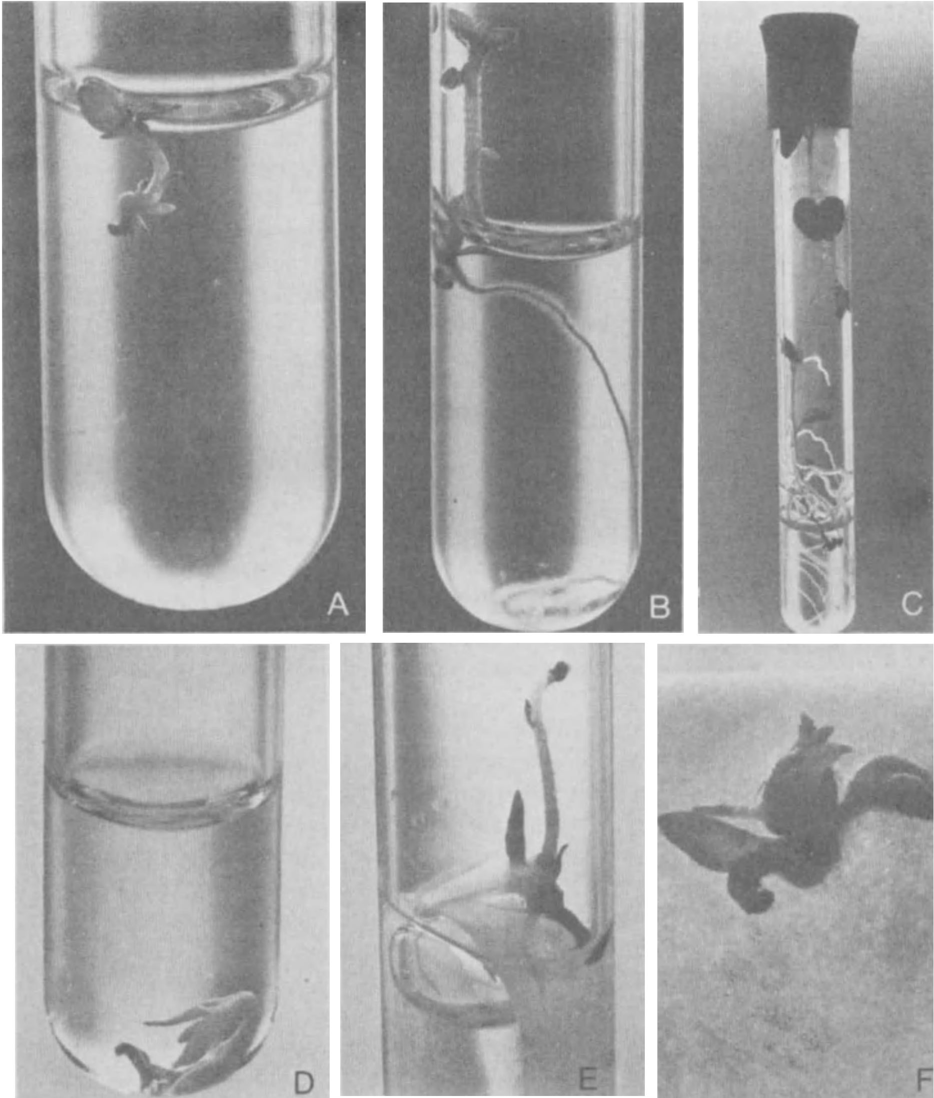

Fig. 1 (A–F). Normal and abnormal development of potato buds in liquid culture. Successive stages during normal development are shown in (A–C). Some buds sink and die unless raised on a filter paper bridge: (D) and (E) show two buds which were comparable before culture (E) was raised. Submerged, tuber-like teratomata (F) which, after transfer to filter paper, is starting to develop into a plantlet

liquid medium are likely to sink and must be supported by a filter paper bridge to prevent submersion and death (Fig. 1D,E). At 23° C growth is even slower but shoots and roots usually develop simultaneously. If the expanding buds become chlorotic, they can be transferred to fresh medium without filter paper bridges. If not transferred, they remain in a state of suspended growth for several months, resuming active growth when moved to fresh medium.

Some rootless shoots that sink in the liquid medium become swollen and distorted, resembling the teratomata as described by PENNAZIO and REDOLFI (1973). These can sometimes be salvaged by using filter paper bridges to raise them above the surface of the liquid (Fig. 1 F).

Rootless shoots developed in culture may also be salvaged by grafting to tomato seedlings. MOREL and MARTIN (1955) found that etiolated plantlets that developed on agar often decayed when moved to soil, but stem cuttings grafted to young tomato plants developed so well that within a month cuttings could be taken which rooted readily. KASSANIS (1967), and HUTH and BODE (1970) found that plantlets could either be planted in soil or grafted onto young tomato plants. The scions survived and grew quickly. MELLOR and STACE-SMITH (1969) speeded the development of rootless shoots by cutting a thin slice from the base, dusting the cut surface lightly with a rooting hormone, and planting the shoot in sand or soil.

2.3.3 Other Factors

Varietal differences sometimes determine the rate of development in culture. QUAK (1961) observed that "some potato varieties do not grow well on media that are quite satisfactory for others". We also found that in many cultivars, a few buds develop a shoot and root within two months of excision while in others, under similar culture conditions, the same development may take 4–6 months, and one or more transfers.

Buds from a single shoot also vary in their development. There seems to be no predictable pattern for rate of development with a single group of buds. Position on shoot and bud size, within the range of 0.3–0.7 mm, appear to have little influence on early rooting. Within 2 months of excision, one or two buds may develop shoot and roots, with leaves of normal colour, whereas others develop several small pale leaves, but without shoot and root. Transferring these pale buds of fresh medium often causes a surge of growth, sometimes within a week.

Once rooted, a plantlet continues to grow without transfer until the culture tube is crowded with roots, and the shoot is coiled at its top. Plantlets in culture can be maintained indefinitely by transferring shoot tips 2–3 cm long to fresh culture tubes at intervals of about 4 months, or when the medium in the old tube is nearly dry. At lower temperature, dessication would be reduced and the transfer interval extended.

3. Virus Eradication

3.1 Factors Influencing Eradication

3.1.1 Antimetabolites

The literature on tissue culture refers to actual and potential benefits of antimetabolites in the culture media. Some experiments have been made on callus cultures (KASSANIS and TINSLEY, 1958); others on stem nodal segments (OSHIMA and

Table 5. Effect of antimetabolites in the culture medium on inhibition and eradication of potato virus X in excised tips

Size of excised tip mm	Antimetabolite	Inhibition	Eradication	Reference
10	malachite green	+	+	NORRIS, 1954
5	malachite green	–	–	THOMSON, 1956
1–2	malachite green	–	–	MANZER, 1958
	thiouracil	–	–	MANZER, 1958
1–2	2,4-dichloro-phenoxyacetic acid	+	+	QUAK, 1961
3–4	malachite green	+	+	VASTI, 1973
	thiouracil	+	+	VASTI, 1973
0.5–0.7	azuguanine	–	–	PENNAZIO, 1973
	fluorouracil	–	–	PENNAZIO, 1973
	thiouracil	+	–	PENNAZIO, 1973
	fluorophenylalanine	–	–	PENNAZIO, 1973

LIVINGSTON, 1961; VASTI, 1973), but most on meristem tips. Some of the antimetabolites, at concentrations that do not destroy the plant, inhibit the multiplication of PVX, but there are few reports of eradication (Table 5). NORRIS (1954) reported that PVX could be eradicated after treatment with malachite green. While most of NORRIS' buds were still infected after treatment, in several, resurgence of the virus was delayed, and one was free from PVX.

VASTI (1973) used meristem tips (3–4 mm long) with malachite green or thiouracil in the medium. When the chemicals were applied separately, all six plantlets on media with malachite green and two on media with thiouracil were PVX-free. When the excised tips were treated with a mixture of malachite green and thiouracil, it was less effective. When PENNAZIO (1973) grew meristems 0.5–0.7 mm long on a medium with thiouracil, PVX remained in all 48 surviving plantlets. VASTI'S high rate of virus eradication from relatively large meristem tips is difficult to explain considering the negative results obtained by other investigators.

Despite the evidence that chemotherapy may assist in virus eradication, the same effect can be achieved more easily and certainly by heat treatment of the infected plant prior to culture of excised buds.

3.1.2 Size of Excised Bud

The size of excised meristem is of utmost importance for determining success particularly for PVX and PVS. KASSANIS and VARMA (1967) excised meristems 0.1 mm long, with or without a leaf primordium and observed that only 20/196 buds developed into plants, but 19 were virus-free. ACCATINO (1966) excised meristems with two leaf primordia and reported that of 18 plantlets, all were free

Table 6. Influence of bud size on potato virus eradication without prior heat treatment

Length	Leaf primordia	Plantlets developed	No. of plantlets from which each virus[a] was eliminated						Reference[b]
mm	no.	no.	LR	A	Y	M	X	S	
0.1		5	5						K and V
		5					5		
		10						9	
0.1–0.25		1				1			K
		3						2	
<2.0		5					5	1	Y and T
	2	18	18	18	18	18	12	3	A
	2–4	19		9			11		G and L
0.12	1	50					24		P and R
0.27	2	42					18		
0.6	4	64					0		

[a] LR, A, Y, M, and S: Potato leaf roll virus, and potato virus A, Y, M, X, and S respectively.
[b] References: K and V: KASSANIS and VARMA, 1967; K: KASSANIS, 1957; Y and T: YORA and TSUCHIZAKI, 1962; A: ACCATINO, 1966; G and L: GREGORINI and LORENZI, 1974; P and R: PENNAZIO and REDOLFI, 1973.

from PLRV, PVY, and PVM, but 6 were infected with PVX, 15 with PVS (Table 6).

KASSANIS and VARMA (1967) observed that different potato cultivars have meristems of different shapes and sizes. We find that buds on a single shoot may vary so much that size is difficult to describe. Designation of bud size in the literature varies: some authors specify total length, others the number of leaf primordia. Either designation gives only an approximate indication of the volume of tissue. In Figure 2, various types of meristems, and the difficulty of specifying the actual size of each, are illustrated. Figure 2A shows an axillary bud with two leaf primordia and four rudimentary leaves. Removal of the rudimentary leaves would leave a meristem with two leaf primordia, with a total length of 0.2 mm. Figure 2B also illustrates an axillary bud with two leaf primordia. If both were left on the meristem, the length would be 0.3 mm; if only one were left the length would be 0.2 mm but the volume of tissue would be considerably smaller than that of the first bud.

Describing the size of the apical meristem is even more difficult. The apical dome (Fig. 2C) is usually wider than it is long, and is surrounded by a whorl of leaf primordia. Despite the larger volume of tissue and the more numerous leaf primordia, we find that the apical meristem is no more likely to root than axillary buds of similar length, and despite its supposedly favourable position on the shoot, it is no more likely to be virus-free. Since it takes longer to excise the apical meristem than other buds, we often discard it.

Figure 2D illustrates an axillary bud from a potato plantlet after several months in culture. There may be up to six such buds on a single plantlet, which though small are remarkably uniform in size and shape. Such plantlets provide uniform material for investigation of meristem development under different conditions of culture.

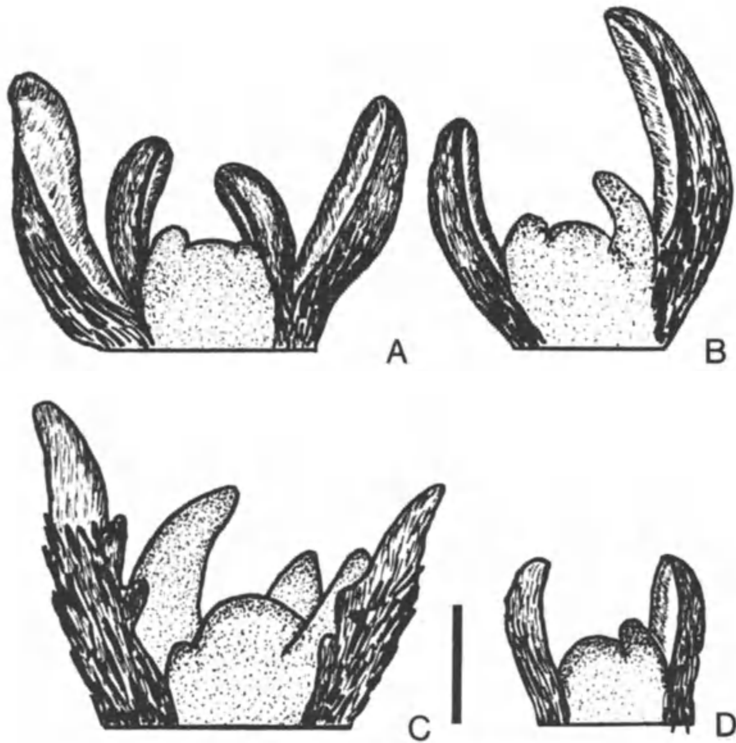

Fig. 2 (A–D). Buds of potato showing the meristematic dome and leaf primordia (*stippled*), and rudimentary leaves (*shaded*): (A) and (B) are axillary buds; (C) is an apical bud showing the larger meristematic dome and more numerous leaf primordia; (D) is an axillary bud from a plantlet after several months in culture. The bar represents 0.2 mm

3.1.3 Heat Treatment

Heat treatment of infected plants before bud excision facilitates the elimination of some viruses that are difficult to eliminate by meristem culture alone (see Chap. V.2 of this Vol.). After heat treatment relatively large buds may be virus-free, and these usually develop readily in culture.

The common potato viruses, listed in order of increasing difficulty of eradication are: leaf roll (PLRV), PVA, PVY, aucuba mosaic, PVM, PVX, PVS, and spindle tuber (PSTV). Most of these include strains which are more resistant to treatment than other. For example, KASSANIS (1950) eradicated PLRV from whole tubers by heat treatment alone, whereas MELLOR and STACE-SMITH (1971) found strains of PLRV that persisted after heat treatment combined with meristem culture.

PVA and PVY are often eliminated by meristem culture without prior heat treatment. MOREL *et al.* (1968) reported that these viruses were eliminated from 85–90% of their meristem cultures whereas PVX and PVS were eliminated from less than 1%, but KASSANIS (1965) found that PVA persisted in 39/39 plantlets developed from buds excised after 12–20 days at 37° C. Other reports indicate

Table 7. Influence of heat treatment and size of bud on eradication of potato virus X (PVX) and potato virus S (PVS)

Material	Heat treatment		Length of bud	Virus eradication		Reference[a]
	Temperature	Period		PVX	PVS	
	°C	weeks	mm	no.	no.	
Tubers	32–35	0–1.5	0.15–0.2	4/6	1/6	McD
		3–13		6/6	6/6	
Rooted cuttings	33–37	0	0.3–0.5	2/9	1/9	S and M
		2–4		8/25	4/25	
		6–8		7/8	5/8	
Mature plants	33–35	0	ca. 0.4	7/59		P
		3		34/42		
		3		2/34		
Rooted cuttings	33–37	0	0.6–1.0	0/32	0/32	S and M
		2–4		3/8	1/8	
		6–8		9/14	1/14	
Sprouted tubers	37	0			15/21	T
		3			16/19	
Sprouted tubers	37	0			23/27	T
		1.5–2			5/6	

[a] References: McD: MACDONALD, 1973; S and M: STACE-SMITH and MELLOR, 1968a; P: PENNAZIO, 1971; T: TAPIO, 1972.

that PVA sometimes persists in plants that have been freed from PVX (CHRISTENSEN, 1968; GREGORINI and LORENZI, 1974).

We found no published information on aucuba mosaic from potato but we have results of a preliminary test of plantlets that developed from meristems excised from heat treated plants and only 4/75 remained infected.

From a plant infected with paracrinkle (PVM), KASSANIS (1957) excised very small buds without prior heat treatment. The single plantlet that developed was virus-free. ACCATINO (1966) found all of 18 plantlets from buds excised without prior heat treatment to be free from PVM. We have eliminated this virus from two cultivars in different tests.In one instance PVM persisted in 3/63 plantlets; in the other in 18/57.

PVX and PVS are seldom eliminated unless the excised buds are very small, or the infected plants are heat treated before the buds are excised. The combined effect of heat treatment and meristem size on elimination of these two viruses is shown in Table 7. MELLOR and STACE-SMITH (1970) compared the relative ease of elimination of PVX and PVS from 21 source plants infected with both viruses. They found PVX was usually more susceptible to heat treatment than PVS, persisting in about 3% of buds excised after 4–6 weeks treatment, and rarely after longer treatment. Occasionally they found more heat tolerant strains of PVX which persisted in nearly 30% of the buds excised after 10 weeks of heat treatment. PVS varied even more than PVX in its resistance to treatment. It was usually more difficult to eliminate than PVX, but from a few source plants there were more plantlets free from PVS than from PVX. There were also cases where

the incidence of PVS-infected plantlets reached its lowest level after 8 weeks heat treatment, but increased again after longer treatment. Šip (1972) also found that prolonged treatment at 37° C was no more effective than 6–8 weeks in reducing the incidence of PVS.

The spindle tuber viroid is the most difficult to eliminate. From plants infected with severe PSTV, Stace-Smith and Mellor (1970) excised buds after 2–14 weeks heat treatment. Severe PSTV survived in 62/66 plantlets that developed, and indexing revealed mild PSTV in the remaining four. One of these four was the source plant in a subsequent series of tests. Buds were excised after 2–12 weeks heat treatment and again the rate of elimination was very low, i.e. only 6/248 plantlets were freed. The variable results of attempted eradication of other viruses suggest that this source of PSTV may have been unusually resistant to treatment, and the viroid from another source might be less difficult to eliminate.

The temperature and duration of heat treatment used by various investigators are shown in Table 7. When the temperature was 37° C the duration of treatment was usually 3 weeks or less, which is about the limit of survival for potato tubers at this temperature. At slightly lower temperatures survival is greatly increased. Mellor and Stace-Smith (1967) tested the survival of tubers, and of plants developed from rooted cuttings, at several temperatures. They found that when air temperature alternated daily from 33 to 37° C and soil temperatures from 30 to 32° C, tubers survived up to 10 weeks and rooted cuttings more than 6 months. After 4 months treatment some PVX-free plants developed from relatively large tissue units, the proportion increasing with longer treatment. They later (1970) compared plants derived from tuber pieces with rooted cuttings as material for heat treatment. On rooted cuttings the shoots that developed during heat treatment were slender, and axillary buds were small, with few rudimentary leaves. Plants developed from tuber pieces had robust shoots, with stout axillary buds, each with many rudimentary leaves to be removed before the buds could be excised. Not only were the buds from cuttings easier to excise, but the total volume was considerably less than buds of similar length taken from plants developed from tuber pieces, and the size difference was reflected in virus eradication. PVS was eliminated from twice as many of the small buds as from stout ones of similar length.

3.1.4 Multiple Infection

Pennazio (1971) obtained conflicting results when he cultured meristems from heat-treated plants of two cultivars. From one, infected with PVX only, 34/42 plantlets were virus-free. From the other, infected with PVY, PVM, PVS, and PVX, only 2/34 plantlets were free from PVX, although all were free from PVY and most from PVM and PVS. He attributed the difficulty of eliminating PVX from this cultivar to the combined infection with four viruses.

Close (1964) studied the influence of several viruses on PVX concentration, multiplication and movement within the plant, in relation to temperature. The concentration of PVX was greater in the presence of PVY than in plants infected with PVX alone, especially at 31° C. At this temperature PVX alone barely multiplied and did not move systemically. The presence of PVY, however, which con-

tinues to multiply and move at 31° C, increased the concentration of PVX by 64 times, and enabled it to establish a systemic infection.

CLOSE'S observations appear to substantiate PENNAZIO'S speculation. Possibly the presence of PVY during heat treatment was responsible for the persistence of PVX even though PVY was later eliminated by excision of small buds. Although PVX has been detected in the meristematic dome of potato buds, there are no such reports for PVY.

PENNAZIO'S results could also be explained on the basis of a heat tolerant strain of PVX. When MELLOR and STACE-SMITH (1970) cultured meristems from heat-treated plants of 18 cultivars the elimination of PVX varied from 54% to 100% according to the source plant. In comparing unknown strains it would be impossible to determine whether the tenacity of the virus in certain cases is due to multiple infection or to intrinsic properties of the particular virus strain.

3.2 Eradication during Culture

The technique of developing virus-free plants by propagating meristems excised from infected plants was based originally on the theory that viruses are unevenly distributed in their host, so that a sufficiently small piece of tissue might be virus-free. There was also evidence that virus concentration was reduced near the shoot tip. Infectivity tests to determine the extent of viral penetration into the apical region were inconclusive until recently. KASSANIS (1967) first suggested that electron microscopic examination of apical meristems might detect the presence of virus particles. APPIANO and PENNAZIO (1972), using thin-section electron microscopy, detected PVX in the cytoplasm of apical dome cells of potato. Since they found the particles in all the meristems they examined, they concluded that the PVX-free plants, occasionally developed by meristem culture, indicated that the virus was eradicated during culture. These observations were confirmed and extended by KRYLOVA *et al.* (1973), by electron microscopy of squash-homogenates of meristems from PVX-infected shoots. They examined meristems of three size groups and detected a few particles in each of 98/100 of those 0.08–0.1 mm; a few more in those 0.1–0.3 mm; and many in meristems 0.3–0.5 mm long. They also examined squash homogenates of meristems after 4 weeks in culture. In cultured apices less than 0.1 mm long they found no particles; in those 0.1–0.3 mm long, they found very few.

The electron microscope observations have been confirmed by direct infectivity tests. Reports to date have been restricted to PVX, but they demonstrate that, if the excised bud is sufficiently small, the virus is inactivated during culture. PENNAZIO and REDOLFI (1974) used sap transmission to detect PVX in potato meristems. They divided meristems of three sizes into two groups: one for direct transmission tests, the other for 90 days culture and subsequent indexing of the resulting plantlets. From the smallest buds, 0.12 mm, there was a highly significant reduction of PVX after culture: 81% of the excised buds were infected; 52% of the plantlets. Among buds 0.27 mm long there was a significant reduction. The largest buds, 0.6 mm long, and plantlets developed from them, were all infected.

The mechanism of virus eradication during culture is not known. INGRAM (1973) suggested that it may be due either to some inactivating factor produced by the explant, or to the effect of some constituent of the culture medium on the virus. QUAK (1972) speculated that the disappearance of virus particles may be attributed to contact of the meristem with the medium.

We believe another theory is even more plausible. Viral replication requires enzymes that are normally available to the cells near the meristematic dome. When small tips are excised, their growth processes are temporarily disorganized and the enzymes that are required for one or more steps of viral replication become unavailable thus interrupting the production of infective virus. In small buds, the degree of disorganization is greatest, and the period of interruption in viral replication is prolonged to the point where viral RNA is degraded and possibly utilized by the plant cells. With large buds there is less disorganization and the enzymes required for viral replication are available before all of the viral RNA is degraded.

3.3 Successful Eradication

3.3.1 Virus Detection

Since the potato is subject to infection by many unrelated viruses, each of which has strains of varying severity, detection and identification of the viruses may be difficult. Sap inoculation, aphid transmission, serology, electron microscopy or electrophoresis may be required. If the only objective is to produce a virus-free clone, it might seem unnecessary to know which viruses were present before treatment, but indexing the source plant will reduce the extent of indexing later in the program. Use of a single source plant, and knowledge of its virus content, indicate which tests must be used to detect any surviving viruses in possibly numerous plantlets. For this reason we reserve a non-treated source plant for indexing. It also serves as check of indicators and techniques when indexing plantlets.

From our experience, PVX and PVS are the most common viruses. To detect these, we use sap transmission tests to *Gomphrena globosa* L. and *Chenopodium amaranticolor* Coste and Reyn. Since the local lesions on the latter indicator induced by PVX appear earlier and obscure, than, those of PVS, detection of PVS in the presence of PVX requires serology. Results of serological tests for PVS are sometimes uncertain and adequate controls must be included.

Serology, or sap inoculation of indicator plants, can be used to detect PVA, PVY, PVM, and aucuba mosaic. DE BOKX (1972) has summarized the test plants commonly used for potato virus detection and identification.

The strains of PLRV that cause symptoms on potato are seldom present in the select stock used for virus eradication. Since PLRV is not sap transmissible, symptomless strains can be detected only by aphid transmissions to *Physalis floridana* Rydb. To detect the very mild strains, uniform seedlings in a controlled environment are necessary (MACCARTHY, 1963). MURAYAMA *et al.* (1973) succeeded in purifying PLRV and preparing a specific antiserum. Serology may eventually be used to detect PLRV in crude sap of infected potatoes.

Table 8. Virus-free potato cultivars developed by meristem tip culture

Cultivar	Reference[a]	Cultivar	Reference	Cultivar	Reference
Aeggeblomme	C	Dunbar Standard	C	Nampa	V
Abnaki	M and S	Early Carmen	N	Netted Gem	S and M
Akebia	C	Early Ohio	V	Nooksack	V
Alamo	M and S	Early Puritan	Ca	Norchief	V
Alaska 114	V	Early Rose	M and M, S and M	Norchip	V
Alma	C	Edelgard	C	Norgold Russet	S and M
Amsel	C	Eigenheimer		Norland	S and M
Aristo	C	Eesterling	M and M, Q	Onaway	V
Arran Comet	K and V	Electra	C	Ontario	V
Arran Consul	V	Elsa	C	Orion	K and V
Arran Pilot	C	Epicure	K and V, S and M	Peconic	V
Arran Victory	K, C	Erstling	C	Pembina Chipper	V
Aryo	C	Essex	C	Piattellina	P
Asparges	C	Fin de Siecle	M and M	Pito	T
Åspotet	C	Forty Fold	V	Primal	M and M
Athene	C	Fundy	S and M	Primula	C
Avon	M and S	Gold Coin	M and S	Pungo	V
Banana	V	Golden Wonder	K and V	Rajke	S
Bea	C	Great Scot	C	Raritan	V
Belle de Fontenay	M and M	Gregor Cups	V	Red La Soda	M and S
Belleisle	V	Green Mountain	N, S and M	Red Pontiac	S and M
Bintje	Q	Gustav Adolf	C	Ricciona	P
Blauwe Eigenheimer	C	Hindenburg	C	Rocks	V
Bona	C	Hochprozentige	C	Rosen	C
Bojar	C	Hudson	V	Royal Kidney	McD
Brennragis	C	Huron	V	Sable	V
Burbank	C	Irene	C	San Michele	G and L
Canus	S and M	Irish Cobbler	K, S and M	Saucisse	M and M
Cariboo	S and M	Jara	S	Sebago	M and S
Carmen	C	Katahdin	M and S, C	Sharpe's Express	K and V
Carnea	C	Kennebec	S and M, C	Snowchip	V
Cherokee	M and S	Keswick	M and S	Superior	V
Chieftain	V	King Edward	Ka, C	Sydens Dronning	C
Chinook	V	Krasawa	C	Tammiston aikainen	T
Clivia	H and B, C	La Chipper	V	Targhee	V
Columbia Russet	M and S	La Rouge	V	Valenciana	Q
Corahila	A	Lilly	V	Warba	S and M
Danshaku	Y and T	Lutzov	C	Waseca	S and M
Dianella	C, Ca	Manota	V	Webbs	C
Dolfis	C	Menominee	V	White Rose	S and M
Dorita	C	Munsterche	C	Yam	V
Drossel	C	Mensa	H and B	York	V
Duke of York	McD	Monona	V	Zitzewitz	C
Dunbar Rover	C	Myatt's Ashleaf	V		

[a]A: ACCATINO, 1966
C: CHRISTENSEN, 1970
Ca: CARLSTRÖM, 1970
G and L: GREGORINI and LORENZI, 1974
H and B: HUTH and BODE, 1970
K: KRYLOVA *et al.*, 1973
Ka: KASSANIS, 1957
K and V: KASSANIS and VARMA, 1967
McD: MACDONALD, 1973
M and M: MOREL and MARTIN, 1955
M and S: MELLOR and STACE-SMITH, 1970
N: NORRIS, 1954
P: PENNAZIO, 1971
Q: QUAK, 1961
S: ŠIP, 1972
S and M: STACE-SMITH and MELLOR, 1968b
T: TAPIO, 1972
V: Vancouver Research Station; not previously published
Y and T: YORA and TSUCHIZAKI, 1962

Potato spindle tuber viroid (PSTV) presents a difficult detection problem. Transmission to tomato seedlings is useful but mild strains remain undetected unless challenge-inoculated with a severe strain (FERNOW, 1967). This test is demanding of both time and greenhouse space, and may not detect every infection. A procedure developed by MORRIS and WRIGHT (1975), involving the extraction of cellular nucleic acids and their separation by polyacrylamide gel electrophoresis, appears to be highly reliable for detecting even mild strains of PSTV.

In treated plants in which a virus is almost, but not quite eliminated, the virus may take much longer to reach detectable levels than it would in a plant newly inoculated with the same virus. MELLOR and STACE-SMITH (1967) reported an instance in which PVX was not detected until 10 weeks after a scion had been grafted onto a tomato seedling, and after five negative tests, although infected scions were usually detected within two weeks of grafting. For this reason, we select a single virus-free plant of each cultivar for propagation and index it at intervals while the stock is being multiplied. At the same time, we maintain several other virus-free plants of the same cultivar in case the first should prove infected.

3.3.2 Virus-Free Cultivars

MOREL and MARTIN (1955) were the first to use meristem culture to eradicate viruses from potatoes. In the two decades since, the technique has been widely applied in many countries, including USA, France, Britain, the Netherlands, Denmark, Germany, Czechoslovakia, Finland, Japan, Italy, and Canada. Virus-free clones are now available for most important cultivars. Table 8 lists these, compiled from published reports and supplemented from our own unpublished data. We recognize that this list is far from complete but it indicates the extent of success.

4. Summary of Recommended Methods

Virus-free plants can be obtained from an infected potato tuber by the following procedure. Plant a single-eyed tuber piece in soil under normal temperature and light. When the first sprout is about 15 cm tall, take a tip cutting 6–8 cm long, remove the two lower leaves, dust the cut surface with rooting hormone, plant the cutting in a 10 cm peat pot of sterilized soil and cover it with a glass beaker for about 10 days. Peat pots are uniform in texture and they assure a soil temperature lower than air temperature during heat treatment, good drainage, and adequate aeration of the soil, all of which contribute to longevity under heat treatment. However, to take another cutting a few weeks later would be helpful in case the first should die during heat treatment. Reserve the parent plant to serve as a check when indexing the plantlets that develop in culture.

Three to four weeks after planting, move the cutting to a growth chamber where the light is 3000–4000 lux for a 16-h day, with air temperature about 36° C during the day; about 33° C at night. Two weeks later, pinch out the tip of the young plant to promote the growth of axillary shoots.

After 6 week's heat treatment, remove an axillary shoot for bud excision, leaving at least two leaves on the plant to encourage further growth. Cut the leaves from the detached shoot, leaving a small piece of each petiole, and enfold the shoot in wet paper to prevent wilting. Sterilization of the shoot is unnecessary, but the buds must be excised under aspetic conditions and the instruments must be sterilized between each bud by dipping first in alcohol, then in sterile water. Under magnification of about 25 ×, excise each bud in turn. Pull back the petiole stump to expose the axillary bud. With a dissecting needle break off the rudimentary leaves leaving only the youngest two leaf primordia (Fig. 2). With a fragment of razor blade in a suitable holder, cut off the meristematic tip, approximately where shown in Figure 2. Use a dissecting needle to transfer the excised bud to the surface of the recommended medium (Table 4). Most of the buds will be 0.3–0.6 mm long, from the cut base to the tip of the longest leaf primordium. Buds smaller than this have a reduced chance of survival; larger buds are more likely to be virus-infected. Maintain the cultures at about 23° C, in 16 h light regime.

Each shoot will provide 8–12 buds, some of which will probably develop into virus-free plantlets. However, some cultivars do not root readily, and some virus strains are unusually persistent. To assure success, take another shoot for bud excision after another 2–4 weeks of treatment. After 2–3 months in culture, transfer buds that have not yet rooted to fresh medium.

When a rooted plantlet reaches 3 cm or more in length, it may be moved to soil, but this is rather a critical point. When first removed from the tube the plantlet is tender and succulent, and will wilt if not quickly protected against loss of moisture. If the soil is carefully watered and the plantlet sprayed with a fine mist, then covered with a beaker for about a week, survival is virtually assured.

Infection in plantlets can usually be detected by preliminary indexing when they are moved to soil. As each is lifted from its tube, one leaf 3–5 mm in diameter, may be transferred to a drop of water in a numbered spot plate. When transplanting is completed, each detached leaf provides inoculum sufficient for one leaf each of the indicators *Gomphrena globosa* and *Chenopodium amaranticolor*. The former develops local lesions typical of PVX within a week of inoculation. The latter shows symptoms of PVX a few days later, or in the absence of PVX, shows local lesions of PVS 10–14 days after inoculation. *C. amaranticolor* infected with PVS often develops systemic symptoms also. Negative results of the early indexing should be confirmed several weeks later by repeated transmission tests or by serology and electron microscopy. We have found that the preliminary indexing detects almost all the PVX and PVS infections.

5. Conclusions

Independent programs have evolved in various parts of the world to eradicate viruses from important potato cultivars. Some viruses can be readily eliminated by heat therapy or meristem culture alone, but for most viruses, heat therapy combined with meristem tip culture is required. The long list of virus-free cultivars developed (Table 8) shows that the techniques of virus eradication have been

extensively and successfully exploited. With new cultivars being released, and with some old cultivars still untreated, potato virus eradication programs will continue for several years.

Neither heat treatment nor meristem culture alters the genetic characteristics of a cultivar. Comparisons of virus-free plants with inoculated plants of the same clone, or with the original, untreated plants, leave little doubt that even symptomless viruses contribute to a decline in productivity. For this reason, new standards of freedom from virus are gradually being incorporated into seed potato improvement programs. In fact, large-scale production of virus-free clones may prove more difficult than virus eradication. The virus-free plants are not immune and, since most potato viruses are readily transmitted, careful sanitation and extensive indexing are required at every stage.

Although meristem culture has been widely applied in developing virus-free cultivars, the full potential of tissue culture has yet to be exploited. The fact that cultured plants can survive for months, almost without attention, suggests that this technique would be useful for long-term storage of important germ plasm (see Chap. VII.3 of this Vol.). Plants in culture require a minimum of storage space, and can be quickly restored to active growth when required. They have the added advantage of complete protection from chance contamination by viruses, or indeed by any organisms. A logical extension of the long-term storage would be the use of cultures for international exchange of virus-free clones. Present quarantine regulations often prohibit exchange, but pathogen-free material should be readily acceptable.

References see page 636.

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Chapter VI

Cell Culture and Secondary Products

1. Applications of Cell Suspension Cultures

H. E. STREET

1. Introduction

An appreciation of the basic techniques of cell suspension culture is essential to any assessment of their realized and potential applications. Following a critical evaluation of the present state of technology in the field, this article outlines the directions in which suspension cultures have contributed to our knowledge of the basic physiology of higher plant cells in culture. Finally, it then attempts to identify the obstácles to further progress and to suggest lines along which development should be pressed if the potential of such cultures is to be further exploited, particularly in relation to increasing our understanding of plant cell differentiation and of the control of specific aspects of cellular metabolism. Current achievements in using callus and suspension cultures for studies on secondary plant products in general and on medicinal compounds in particular are outlined in some detail in the articles written respectively by BUTCHER (Chap. VI.2 of this Vol.) and STABA (Chap. VI.3 of this Vol.). The concluding section of this article does, however, consider how far advanced techniques of handling cell suspension cultures open up new approaches to the study of secondary product biosynthesis in plant cell cultures.

2. Techniques of Cell Suspension Culture

2.1 Batch Cultures on Platform Shakers

Suspension cultures are normally initiated by transferring pieces of undifferentiated callus to a liquid medium which is agitated during incubation. Successful establishment of the suspension culture depends upon the initial callus being friable and it may be necessary to use an appropriate level of phytohormones in the callus culture medium to achieve the necessary friability of the callus. During the first incubation passage in the liquid medium some cells are released from the explants and multiply. For subculture, a pipette or syringe with a sufficiently fine orifice should be used to exclude any persistent large cell aggregates derived by only partial break-up of the initiating callus fragment. The suspension transferred on subculture should consist of free cells and small cell aggregates. The culture medium used for suspension culture is usually based upon that which maintains good growth of callus; it may, however, be necessary to modify this medium (particularly its phytohormone content) to achieve a high growth rate and good cell separation in liquid medium.

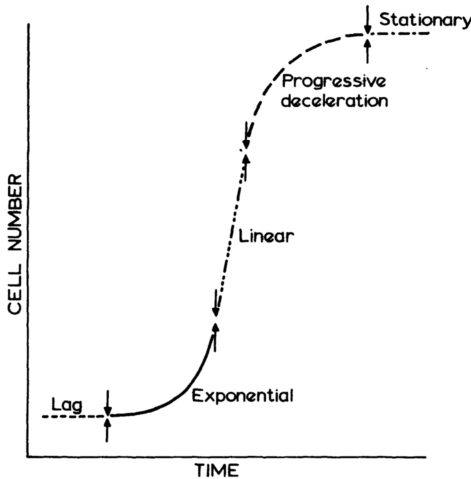


Fig. 1. Model curve relating cell number per unit volume of culture to time in a batch-grown plant cell suspension culture. Phases of the growth cycle labelled. After WILSON *et al.* (1971)

Platform (orbital) shakers are widely used for the initiation and serial propagation of plant cell suspension cultures. They should have variable speed control (30–150 rpm) and the stroke (throw) should be in the range 2–4 cm orbital motion. The shaker should be sited in an air-conditioned room (with good temperature control within the range 24–30° C) or the platform should be enclosed in an incubator cover. The platform is fitted with clips to carry the chosen culture vessels. Fluorescent light can be mounted above the platform and cultures to be incubated in darkness wrapped in aluminium foil.

Wide-mouthed Erlenmeyer borosilicate flasks are widely used as culture vessels; they can be modified in a number of ways to control the gas phase composition of the cultures (RAJASEKHAR *et al.*, 1971). The flasks are normally sealed with aluminium foil. The volume of culture should be appropriate to the culture vessel, e.g. 20 ml per 100 ml and 70 ml per 250 ml flask.

Such *batch* cultures (cultures in a fixed volume of culture medium) increase in biomass by cell division and cell growth until a factor in the culture environment (nutrient or oxygen availability) becomes limiting. The cells then enter a stationary phase during which cell dry weight declines; the stability of the cells in stationary phase depends upon the species and on the nature of the growth-limiting factor (cells brought to stationary phase by nitrogen limitation retain viability longer than when carbohydrate-starved). When such a stationary phase cell suspension is subcultured, the cells in succession pass through a lag phase, a short-lived period of exponential growth, a period of declining relative growth rate and then again enter stationary phase (Fig. 1). Traditionally such cultures are at each subculture initiated from a relatively high cell density and the cells therefore accomplish only a very limited number of divisions (cell number doublings) before again entering stationary phase. For example, suspension cultures of English sycamore (*Acer pseudoplatanus* L.) initiated at ca. 2×10^5 cells/ml will, in the basic culture medium of STUART and STREET (1969), reach a final cell density of ca. 3×10^6 cells/ml corresponding to not more than four successive doublings of the initial population. Cultures can, however, be initiated successfully from lower

initial densities (in the case cited above from not less than 1.5×10^4 cells/ml) and in such cultures the period of exponential growth (period during which the specific growth rate is constant and maximal) is correspondingly extended. For any cell culture strain and culture medium combination there is a minimum inoculation density below which the culture will fail to grow on subculture. For instance in the case of sycamore cultures the minimum initial density can be lowered to 2×10^3 cells/ml by using a more complex medium (STUART and STREET, 1971) and still lower (down to 600 cells/ml) by appropriate enhancement of the CO_2 content of the gas phase (results of MANSFIELD quoted by STREET, 1973a, GATHECOLE *et al.*, 1976).

2.2 Large-scale Batch Cultures

Large scale (4.5 l cultures in 10 l borosilicate bottles) batch cultures in which aeration and dispersion of the cells is achieved by spinning the culture vessels (80–120 rpm at an angle of 45° to the horizontal) have been described by LAMPORT (1964) and SHORT *et al.* (1969a) and used for studies on growth kinetics and on nucleic acid metabolism. By achieving adequate gaseous exchange and dispersion of the cells by forced aeration alone (TULECKE and NICKELL, 1960; TULECKE, 1966, GRAEBE and NOVELLI, 1966; KURZ, 1971) or aeration combined with internal magnetic stirring (MILLER *et al.*, 1968; VELIKY and MARTIN, 1970; WILSON *et al.*, 1971) it has been possible to devise more sophisticated batch culture systems and from these to develop both closed and open continuous culture systems.

Such batch culture systems not only scale up cell and culture medium yield for detailed biochemical analysis but enable the progress of growth and metabolism to be monitored within the same culture vessel by frequent manual or automatic sampling. Although the nutrient supply is primarily determined by the fixed culture volume, it is possible during the progress of growth in such batch cultures to alter at any desired time the physical environment (temperature, gaseous exchange, illumination) or to add as a concentrate (hence in small volume) individual nutrients, growth factors, phytohormones or inhibitors. Particular interest might apply to changes in the culture environment (physical and nutritive) effected as the culture approaches or enters the stationary phase of its cell division cycle. Such experiments would be justified on the grounds that there is evidence that subsequent differentiation may be determined by conditions operating during the period of cell multiplication (meristematic phase) (FOSKET and TORREY, 1969) and that the production of many secondary plant products normally occurs in non-dividing cells, often in non-dividing cells which have embarked upon a special pathway of differentiation. There is, however, no published work known to the author in which large-scale batch suspension cultures have been subjected to such "sequential" treatments (KENT and STEWARD, 1965) involving a stage during which the culture is maintained in a non-dividing state by depletion of a cell division factor or presence of a cell division suppressor.

We do not yet know whether particular pathways of differentiation have their origin from cells arrested in division at particular points in the cell cycle, but it is well established that important changes in gene expression occur during this cycle

(MITCHISON, 1971). It is therefore of considerable interest that recent studies using rapid-automatic sampling of batch-propagated cell suspensions have shown that such cultures can show a high degree of division synchrony persisting through up to five successive cell generations (KING and STREET, 1973; KING *et al.*, 1973). Such cultures are being used currently to analyse the sequence of biochemical events which intervene between successive cell divisions (KING *et al.*, 1974; GOULD and STREET, 1975). It is also clear that the nature of the limiting nutrient which brings the cells into stationary phase determines the stage in the cell cycle at which they are arrested (GOULD, 1975). Further studies with such synchronous cell cultures controlled in growth rate by particular limiting nutrients or by different external phytohormone levels may therefore lead to a deeper understanding of how the conditions operating during cell division can exert a controlling influence on gene expression in division-arrested cells.

2.3 Closed Continuous Culture Systems

A *closed* culture is one in which the cells are retained so that there is progressive increase in cell density whilst growth continues—the batch cultures described above are closed systems. A *closed continuous* system is one in which nutrients in excess of the culture requirements are supplied by continuous inflow of fresh medium and this is balanced by continuous harvesting of *spent* medium. A system of this kind has been described for the culture of plant cell suspension cultures (WILSON *et al.*, 1971; STREET *et al.*, 1971; STREET, 1973b) (Fig. 2, 3). The essential

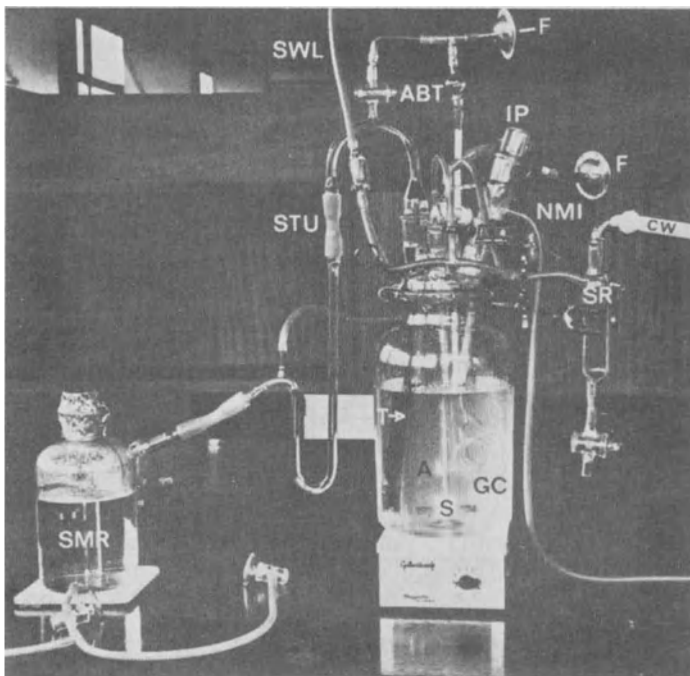
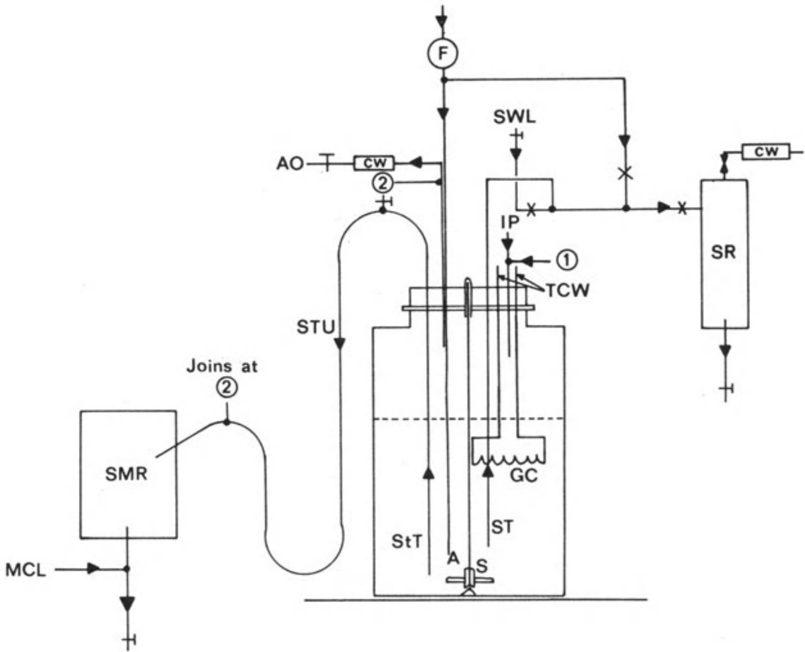


Fig. 2

feature of this is the wide stilling tube (2.5 cm internal diameter) and associated siphon. The medium moves up the stilling tube at a slow rate and hence is free from cells when it moves over the apex of the siphon section (gravity separation of cells from spent medium). This system was used by WILSON *et al.* (1971) to prolong exponential growth and achieve a very high cell density (2×10^7 sycamore cells/ml). The culture reached such a high viscosity that it caused failure of the magnetic stirrer and oxygen limitation of growth.

Such a system would seem to be particularly suited to imposing the "sequential" treatments, referred to above under batch cultures, since the flowing medium establishes a steady state composition of the "spent medium" which can be rapidly altered and monitored. This enables the rate of consumption of a nutrient or metabolic precursor substance to be calculated at a known external concentration within the culture. However, what has not been critically tested is how far suspension culture populations (of predetermined growth history) can then be maintained stable and non-dividing in such a system. If such "maintenance" cultures



Figs. 2 and 3. A *closed* continuous culture system for growth of plant cell suspension cultures. Fig. 2. Complete assembly. Fig. 3. Flow diagram. Key (1): medium input *A*: aerator (No. 2 aerator). *AO*: air inlet (Fig. 3 only). *ABT*: air bleed tap for removal of air which may become trapped in the stilling tube (Fig. 2 only). *CW*: cotton wool filter. *F*: miniature air line filter. *GC*: glass coil through which circulates water at a controlled temperature (*TCW*). *IP*: socket for introduction of cell suspension. *NMI*: input of new medium (Fig. 2 only). *S*: PTFE-coated magnetic bar activated by the magnetic stirrer. *SMR*: spent medium reservoir from which spent medium may be collected aseptically (line for wash out of tube and tap with mercuric chloride shown in Fig. 3: *MCL*). *SR*: sample receiver. *SWL*: sterile water line to wash out *SR*. *ST*: sample tube (labelled in Fig. 3 only). *STU*: siphon tube unit. *StT*: stilling tube up which spent medium rises slowly and is thereby separated from the cells of the culture. (2) after STREET *et al.* (1971), (3) after WILSON *et al.* (1971)

can be stabilised, it may be possible to induce them to embark upon desired pathways of differentiation by appropriate nutrient-phytohormone combinations. The possible importance of working with such maintenance cultures is illustrated by work showing that accumulation of secondary products in cell suspensions may only occur when growth is restricted by nutrient depletion (Nash and Davies, 1972, Hahlbrock, 1972) and that there exists an antagonism between cell division and such an indicator of cytodifferentiation as the development of functional chloroplasts (Davey *et al.*, 1971).

There are now a number of instances where not only primary metabolites (e.g. amino acids, nucleotides) but also secondary products produced by cell suspension cultures have been shown to be released in significant amounts into their culture medium. As examples may be mentioned coumarins (Reinhard *et al.*, 1968a; Brocke *et al.*, 1971), lignins (Carcellar *et al.*, 1971), polysaccharides (Olsen, 1971; Carcellar *et al.*, 1971), monoterpene derivatives (Macrae and How via Jones, 1974) and paniculides (Butcher and Connolly, 1971). In such cases a maintenance culture in a closed continuous system should enable the chemical product to be continuously harvested from a fixed culture biomass. This would discount the objection sometimes raised to higher plant cell cultures as potential commercial biosynthetic systems in that they are very slow growing compared with micro-organisms. If a large biomass could function to release, over a long period, a secondary plant product or a desired derivative from a supplied precursor, then the time required to grow up the biomass would be of small economic consequence. Further, the ability in the closed continuous system of constantly removing the released metabolite could mimic a biological "sink", preventing any feed-back inhibition of synthesis. It would also stop the product from exerting any "staling" effect (toxicity due to the action of the product at the exposed surfaces of the cells) by preventing its accumulation.

The observation that metabolites very different in chemical nature and in molecular weight can be released by viable cells into their culture medium raises the possibility that "leakage" mutants might be found for a still wider range of cell products. If cultured cells synthesise and retain a chemical which can be readily detected by its absorbance or fluorescence or by a color or precipitation reaction with a non-toxic reagent, it should be possible to detect such "leakage" mutants by single cell cloning (Street, 1973a) and analysis of the solidified medium zones immediately surrounding the cell colonies.

2.4 Open Continuous Culture Systems

Open continuous systems involve regulated new medium input and balancing harvest of an equal volume of *culture*. They are of particular interest to the present discussion because they allow the establishment of steady states of growth and metabolism, study of the changes which occur in transitions from one steady state to another, and the identification of the controlling factors. Such open systems may be either *chemostats* (in which continuous new medium input is set at a predetermined rate and determines the nature of the resulting equilibrium) or *turbidostats* (in which cell density is set at a predetermined level—as monitored by

the optical properties of the culture—and new medium is called for intermittently to maintain cell density within limits controlled by the sensitivity of the photocell control unit).

The chemostat system is particularly adapted to the study of steady states where the specific growth rate (μ) of the cells is below the achievable maximum specific growth rate (μ_{max}) for the particular cell line, culture medium and physical conditions of culture adopted. Stable cell densities are achieved in a chemostat as a result of an equilibrium established between the dilution rate (D) $\left(\frac{\text{flow rate/day}}{\text{culture volume}}\right)$ (which determines the supply of the *limiting* nutrient to the culture) and the mean generation time (g) of the cells where D and μ are numerically equal. The relationship between μ and g is $\log_e 2/g = 0.69/g = \mu$. By raising the flow rate and hence the dilution rate and supply of the limiting nutrient, a new, uniform, and faster specific growth rate and a new, fixed, and lower cell density results. Eventually, if dilution rate is progressively increased, we reach the critical dilution rate which is in equilibrium with μ_{max}. Any further increase in dilution rate leads to "wash-out" of cells. Hence chemostat cultures are operated safely only below this critical dilution rate.

The turbidostat system is, in contrast, particularly valuable for work at low cell densities where growth approaches or is at μ_{max}. There is no problem of wash-out but if operated at high cell density (and hence at low growth rate) the culture increasingly tends to "close down" (cease growth).

The 5 l batch culture vessel developed by WILSON *et al.* (1971) has been modified to function as the vessel for chemostat and turbidostat systems by the addition of (1) a constant level device, the electrodes of which operate the solenoid valve controlling harvesting of the culture, and (2) a loop through which the culture is circulated (by a peristaltic flow inducer) external to the main culture vessel, from which it is harvested and in which its optical properties can be monitored. It was the demonstration that, at each steady state in chemostat operation, the optical properties (light absorption or scattering), as monitored in the flow-through cell in the loop, were very constant, that enabled the system to be converted to a turbidostat. In this system the density monitor (light source, flow-through cell and photocell) sends signals to an electronic control circuit which controls a medium input solenoid valve. The flow diagram of the turbidostat system of WILSON *et al.* (1971) is shown in Figure 4. A more recent account of the theory of these continuous systems and of their commissioning is presented by KING and STREET (1973).

These systems have been examined mainly with a sycamore cell suspension but can be successfully operated with other well dispersed suspension cultures. However, with suspensions of a number of species (e.g. *Atropa belladonna*, *Daucus carota*, *Rosa* sp.) a significant degree of cell breakage has been observed due to circulation of the culture through the external loop by means of the flow inducer. In such cases successful chemostat cultures have been established by omitting the external loop and collecting excess culture through a single tubulure (inserted at the same level below the surface of the culture as those used to operate the external loop) maintained free of cells in the intervals between culture harvests by a slow stream of sterile air. A two-way solenoid valve admits air until the signal

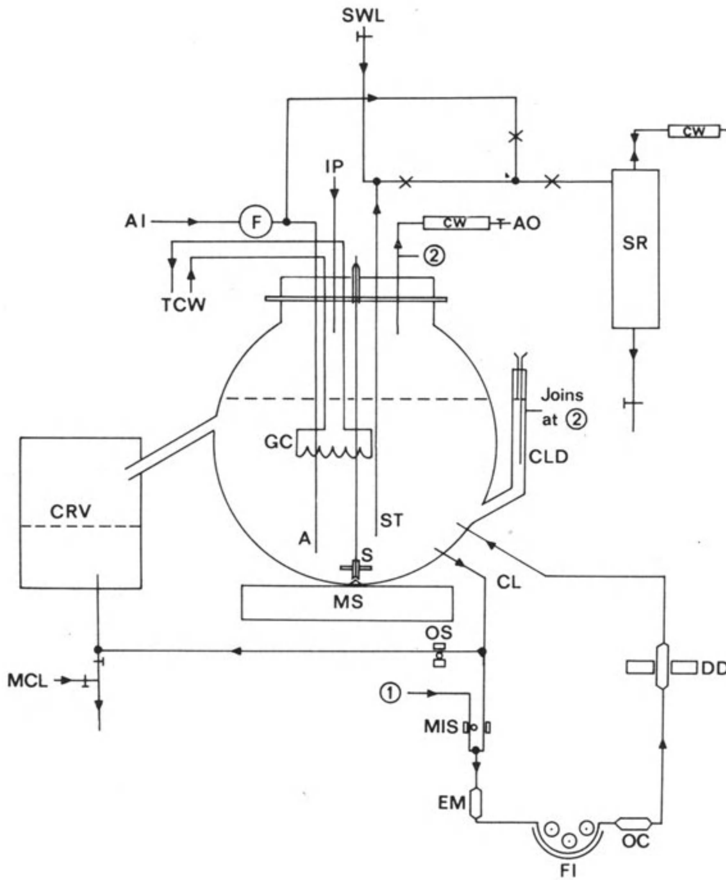


Fig. 4. Flow diagram of an open continuous culture system—a turbidostat. Key *AI*: air inlet. *A*: aerator. *AO*: air outlet. *IP*: socket for introduction of cell suspension. *F*: miniature air line filter. *CW*: cotton wool filter. *GC*: glass coil through which circulates water at a controlled temperature *(TCW)*. *SR*: sample receiver. *SWL*: sterile water line to wash out *SR*, *ST*: sample tube. *S*: PTFE-coated magnet bar activated by the magnetic stirrer *(MS)*, *CLD*: constant level device which operates the outlet solenoid valve *(OS)* through which culture is harvested to the culture receiving vessel *(CRV)*. *MCL*: mercuric chloride solution entry to wash out tap and tube through which excess culture collected from *CRV*. *CL*: circulation loop via which culture is passed to the density detector *(DD)*, observation chamber *(OC)* via the flow inducer *(FI)*. *EM*: electrode module permitting insertion of pH electrode and/or oxygen or CO_2 electrode in the circulation loop. *MIS*: medium input solenoid operated by *DD*. (After WILSON *et al.*, 1971)

from the constant level device closes the air inlet valve and opens the culture harvesting valve. This is immediately reversed once the electrode of the constant level device is again free of culture.

The development of continuous culture systems specially designed for long-term aseptic operation and for handling higher plant cell suspensions has added a

new experimental dimension which is only now beginning to be exploited and whose potential in the field of cell physiology is not yet fully appreciated. To introduce this subject it is necessary to remind ourselves of the patterns of growth and metabolism which have been observed in batch cultures and to see how these relate to the steady states achieved in open continuous culture systems.

3. Growth and Metabolism of Plant Cells in Batch Culture—Cytodifferentiation

The basic pattern of increase in cell number per unit volume in batch culture is illustrated in Figure 1. During lag phase the stationary phase cells of the inoculum embark upon a massive synthesis of new cytoplasm and associated organelles, replicate their DNA and then begin to divide. Then for a strictly limited period cell division rate is constant and maximal (μ_{max}). During this period the mean cell volume declines sharply. Following this the growth begins to decline (slowly at first and later at an ever-increasing rate), mean cell volume increases, and the cells move into a stationary phase. If during this pattern of growth we assess, on a per cell basis, physiological activities (e.g. respiration), levels of individual cell constituents, and the activities of individual enzymes, we can trace the emergence and decline of a succession of metabolic patterns. Thus in sycamore cell suspensions, RNA synthesis is initiated prior to cell division, proceeds for a time at a greater rate than the cell number increases, and then ceases whilst cell division is still proceeding (SHORT *et al.*, 1969b; NASH and DAVIES, 1972). Free nucleotides (in sycamore cells predominantly UDP-glucose and ATP) are synthesised rapidly during lag phase, presumably an essential preparation for subsequent synthesis of cell-wall polysaccharides and as an energy source for the energy-requiring processes involved in cell division; net synthesis of nucleotides ceases very shortly after the onset of division (BROWN and SHORT, 1969). Similar transient high activity in carbohydrate oxidation by the pentose phosphate pathway during lag phase has been interpreted as providing the necessary NADPH for the high level of biosynthesis characteristic of this phase of the growth pattern (FOWLER, 1971). In cell cultures of sycamore (MACKENZIE and STREET, 1970) and of a range of other species (LARUE and GAMBORG, 1971) a very sharp peak in ethylene production occurs late in the cell division phase when the cells are beginning to increase in mean cell volume. Large changes in the activity of the enzymes phenylalanine ammonia-lyase (PAL) and in p-coumarate: CoA ligase occur immediately prior to stationary phase in cultures of *Glycine max* (HAHLBROCK *et al.*, 1971). A similar peak of PAL activity has been reported in cell cultures of *Rosa* sp. (DAVIES, 1972). These changes coincide with maximum production of total phenols by the cultures. The production of other secondary products by cell cultures has been shown to be most active during a restricted phase of the growth cycle; such production is often markedly influenced in its intensity by the phytohormone composition of the culture medium (CARCELLAR *et al.*, 1971; KAUL and STABA,

1967; OGUTUGA and NORTHCOTE, 1970; TABATA *et al.*, 1971). Thus during the progress of batch culture, cultured cells pass through a series of contrasted physiological states which encompass cells in transition to a meristematic state; cells expressing high meristematic activity, cells undergoing expansion and becoming either metabolically quiescent or geared to express certain restricted metabolic pathways.

Although cultured cells do not correspond precisely, either structurally or physiologically, with particular tissue cells of the plant body, is it legitimate to consider that the changes in physiological activity observed during the growth cycle of batch cultures are of the same nature as those which are involved in normal cytodifferentiation? Animal physiologists define cytodifferentiation as involving relatively permanent and irreversible changes in gene activation expressed in terms of the synthesis of enzymes and other cellular proteins. More minor and readily reversible changes in physiology considered to reflect the operation of allosteric and other "fine" processes of metabolic regulation, were termed "modulations" to distinguish them from cytodifferentiation. Certainly the transient nature of the changes discussed above in relation to batch cell cultures emphasises their reversibility. However, the very concept of totipotency as it has been developed from plant tissue culture studies indicates that the changes involved in plant cytodifferentiation (provided they have not proceeded to the point where cells senesce and die) are fully reversible; that living differentiated plant cells can, under appropriate stimuli, again return to the condition of the initial zygote. Further, even where, in callus induction, the achievement of a capacity for active cell division in the explant cells is not correlated with the achievement of totipotency (capacity for plant regeneration), the cells appear to have lost any specific characteristics associated with the organ (root, shoot, leaf) from which they were derived.

Turning back to the distinction drawn between cytodifferentiation and modulation, it is interesting to note that in a recent work on differentiation in animal cells (TRUMAN, 1974) it is conceded that "it is not at all easy to draw a distinction between those enzymes which are fundamental to differentiation and those which represent very short term modulations in the activity of cells." The same author in considering cytodifferentiation in liver cells states that "differentiation and modulation do not represent distinct processes but are merely the extreme ends of a spectrum of changes that can occur."

The question posed above regarding the equivalence with the processes of normal cytodifferentiation of the physiological changes observed in cell suspensions, in response to their changing culture environment in batch culture, cannot be properly resolved until a normal pathway of tissue cell differentiation can be exactly and reproducibly induced in cultured free cells. Meanwhile it should be borne in mind that a particular pathway of differentiation may only in part or not at all depend upon changes in the transcriptional activity of special sets of genes. It could be the outcome of the influence of cytoplasmic factors in the stability and transport of RNA species or other aspects of the translational steps in gene expression. Meanwhile it is urgently necessary to resolve how far changes in translational controls can account for the observed physiological changes in cultured cells.

4. Steady States of Growth and Metabolism Achieved with Open Continuous Culture Systems

Studies on batch cultures showed that even during the limited period of exponential growth (constant specific growth rate), cell division was not coupled to cell dry weight and protein content so that, even during this phase of the growth cycle, the cells were changing in size and chemical composition (KING and STREET, 1973). The development of the continuous culture systems described above, and particularly of the chemostat system, enabled two questions to be experimentally investigated: (1) could continuing balanced growth (the cells remaining constant in growth rate *and* in chemical composition and physiological activity) be achieved if the cells were kept in a constant culture environment? (2) would it be possible to stabilise cells in the otherwise transient states observed in batch culture and could the particular physiological state which was desired be chosen by predetermination of the equilibrium growth rate in the chemostat?

Work with sycamore cell cultures, using a single culture medium (STUART and STREET, 1969) has shown convincingly that long-term steady states can be achieved (KING and STREET, 1973; KING *et al.*, 1973). The balanced state of the cells was characterised by determinations first of cell number, packed cell volume, cell dry weight, contents of protein, DNA and RNA and respiration rate. Further work has shown cells in such steady states to have a constant rate of nitrogen assimilation (YOUNG, 1973), constant levels of free amino acids (STREET *et al.*, 1975) and of activity of enzymes concerned in nitrogen assimilation (YOUNG, 1973), and in carbohydrate respiration (FOWLER and CLIFTON, 1974). When such steady states are established in the chemostat, the medium also becomes of constant composition (as illustrated by constancy of pH and of levels of glucose, phosphate and nitrate).

When a population of sycamore cells growing in the medium of STUART and STREET (1969) (in which the limiting nutrient is nitrogen) is taken through a series of steady states (by stepwise alteration of the dilution rate), it can be shown that if the dilution rate is then returned to that of an earlier steady state, the cells again achieve not only the new predictable growth rate but also the physiological and morphological characteristics earlier recorded for that steady state. In such an experiment the cytological and physiological changes, which can be induced by changes in the culture environment, are fully reversible and the nature of each steady state is determined by the equilibrium culture environment. Interest will now focus on detailed studies of the kinetics of the transitions between steady states, particularly in terms of changes in the levels of individual enzymes and structural proteins and in all aspects of RNA metabolism. Such studies will clearly advance our knowledge of metabolic regulation in higher plant cells and may lead to a better understanding of the processes involved in cytodifferentiation.

Just how far all the transient metabolic states observed in batch culture could be stabilised by chemostat culture is at present uncertain. Even within a limited range of experiments involving a strain of sycamore cells and modifications of the nitrogen, phosphate, and carbohydrate supply of a basic medium, steady states

Table 1. Analytical data from chemostat cultures of *A. pseudoplatanus* L. cell suspensions, steady states established at different dilution rates (growth rates)

Limiting nutrient	Analytical data				Reference
Nitrate	Dilution rate (per day)	0.11	0.16	0.19	KING and STREET (1973)
	Dry weight (mg 10^6/cell)	1.9	1.7	1.5	
	Cell volume (µl 10^6/cell)	52	43	43	
	RNA (µg 10^6/cell)	42	60	71	
Phosphate	Dilution rate (per day)	0.14	0.22	0.39	WILSON (1971)
	Total "Folin" phenolics	27.4[a]			
	(µg gallic acid	49.0[a]	11.5	7.7	
	equiv. 10^6/cell)	79.0[a]			
Nitrate	Dilution rate (per day)	0.09	0.15	0.19	FOWLER and CLIFTON (1974)
	Activities[b] of 6-phospho-gluconate dehydrogenase	21.0	19.5	42.0	
	Pyruvate kinase	23.0	22.0	38.0	
	Phosphofructokinase	9.0	10.0	7.0	
Nitrate	Dilution rate (per day)	0.09	0.14	0.21	YOUNG (1973)
	Activities[b] of γ-glutamyltransferase	280	200	305	
	Nitrate reductase	0.24	0.32	0.32	
	Glutamate dehydrogenase	6.45	5.14	9.46	

[a] At this dilution rate level of phenolics was still rising when the experiment ended although the culture was in a steady state of growth.
[b] n moles substrate or product converted per min per 10^6 cells.

widely different in cell cytology and metabolism have already been described (Tables 1 and 2). Such experiments could be extended (1) by making each essential nutrient in turn the limiting nutrient and (2) by alterations in the growth factor and phytohormone additions. This would clearly extend the range of cellular variations which could be stabilised. Superimposed upon this range of "nutrient" environments could be changes in the physical parameters of the culture system and in its gas exchange relationships. Such a wide and systematic study could lead to dramatic amplification of the enhancement or suppression of particular metabolic pathways.

The turbidostat system offers a simplified system for study of the effect of physical factors and growth-regulating substances on growth rate and associated metabolism because the culture can be operated under non-limiting nutrient conditions (at μ_{max} for the particular basic culture medium chosen). Currently the most actively growing cell suspension cultures have a doubling time significantly below that achieved by the cells of organised meristems. The most rapidly growing suspension culture strain of sycamore has a doubling time of ca. 40 h. Cells in the root meristem of sycamore seedlings have a value below 22 h. Definition of the cultural conditions capable of supporting meristematic activity in culture equal to that ever achieved within the whole organism could be attempted with the turbi-

Table 2. Influence of the nature of the limiting nutrient on the steady state composition of the cells of *A. pseudoplatanus* suspensions in chemostat cultures at similar dilution rates (and hence growth rates)

Culture reference	A	B	
Limiting nutrient	nitrate	urea	
Dilution rate (per day)	0.14	0.13	YOUNG (1973); STREET *et al.*, (1975)
Dry weight (mg 10^6/cell)	1.6	5.1	
Cell volume (µl 10^6/cell)	46	186	
Protein (µg 10^6/cell)	341	1154	
Culture reference	**C**	**D**	
Limiting nutrient	nitrate	glucose	
Dilution rate (per day)	0.15	0.15	Unpublished data of P. J. KING (Univ. Leicester)
Dry weight (mg 10^6/cell)	1.84	0.55	
Cell volume (µl 10^6/cell)	53	33	
Starch (µg 10^6/cell)	228 (12)[a]	87 (16)	
Protein (µg 10^6/cell)	427 (23)	369 (67)	

[a] Figures in brackets = % of cell dry weight.

dostat system. In the course of such experiments it would be possible to determine the affinity of the uptake mechanisms for phytohormones and to construct balance sheets showing the fate of absorbed labelled phytohormone molecules (KING, 1976). Identification, via the turbidostat system, of the conditions permitting expression of the genetically-determined maximum rate of cell division would provide the basis for determining, via synchronously dividing batch cultures, what aspects of the cell cycle are controlled in rate by phytohormones. The turbidostat could also be used to investigate, in cultures achieving similar high rates of biomass production, the phytohormone control of the balance between cell division and cell expansion.

The above general considerations of the experimental potential now available to plant cell physiologists via the growth of plant cells in continuous culture systems, form the essential basis for considering their potential applications in the investigation of secondary product biosynthesis.

5. Applications of Continuous Culture Systems in Secondary Plant Product Biosynthesis

BUTCHER in Chapter VI.2 of this Volume draws our attention to the different situations pertaining to secondary plant product biosynthesis within whole plants. These range from products widely distributed in the plant kingdom and whose synthesis is not confined to specialised cells or cell groups, to those characteristic of particular families, genera or species and whose synthesis takes place in

localised and highly specialized cells or cell groups. As might be expected, compounds of the first group (e.g. coumarins, particular flavonoids, phytosterols) either identical with or chemically related to those of the whole plant have been frequently detected in plant tissue and cell cultures (PUHAN and MARTIN, 1971). In contrast, compounds at the other end of the range have, with few if any exceptions, only been detected in tissue cultures in which a high level of organisation (cytodifferentiation or organ initiation) has developed (RAJ BHANDARY *et al.*, 1969; THOMAS and STREET, 1970; TABATA *et al.*, 1972). It can therefore be argued that progress towards inducing particular specialised patterns of cytodifferentiation in cell cultures will need to be achieved before they can be utilised for the study of the synthesis of volatile oils, resins, latex constituents and so on. Certainly the most promising immediate application of continuous cell cultures in the synthesis of secondary plant products would be expected to come from work with appropriately highly dispersed cell suspensions in which the desired class of compounds can be demonstrated even if currently at very low yield.

Where a desired secondary product or particular plant enzyme is only or most actively accumulated during the later stages of batch culture (when the specific growth rate is declining) then it should be possible, by chemostat culture, to stabilise the cells at the growth rate consistent with the highest cellular content of the product. Since in batch culture the cells rapidly pass through this point in their cycle of growth and proceed into stationary phase where either production is suppressed or the cells lose viability, it may be that very much higher levels of product will be built up in the steady state cells than is ever achieved in batch culture. Furthermore, such a steady state should form the base line from which to make further perturbations in the culture environment to see if these further amplify the biosynthesis. Once the highest level of accumulation consistent with the maintenance of cell viability has been achieved, it will be possible to harvest the product either continuously (continuous run-off of surplus culture) or as a single harvest of the whole culture. This latter course would probably be indicated where a sufficiently slow growth rate is necessary for high product accumulation. Work on protein production by chemostat cultures (KING and STREET, 1973) indicates, however, that it may be important to consider not only the level of product per unit volume of culture but the total output of product per unit time (equilibrium level of product × dilution rate). Maximum protein yield occurs at a growth rate not coincident with that for maximum protein content per cell. It may also be important to look at the effective yield of product per unit of the more expensive constituents of the culture medium, e.g. yield of product per unit of sucrose or organic nitrogen or specific precursor supplied at the chosen dilution rate.

In cases where maximum levels of a secondary product (e.g. visnagin by *Ammi visnaga*—KAUL and STABA, 1967) or of an enzyme (e.g. threonine deaminase by *Rosa* sp.—DAVIES, 1971) develop early in the growth cycle of batch culture (either during or at the beginning of the decay of exponential growth), then experimentation with a turbidostat system is indicated. This would enable the influence of growth regulating substances, physical factors, and the feeding of precursors to be studied under non-limiting nutritional conditions. Here a relatively high proportion of the total culture volume will be running to harvest per unit time; at the

same time a high proportion of the nutrients supplied will also be running to waste. It would here again be important to consider the conditions most appropriate to economic production—this again might be at a culture growth rate below that at which the product is at its highest attainable level per cell.

The assumption in the above discussion has been that very significant amplification of secondary product production could be achieved with selected species of cells (and hence for selected products) by exploiting the readily reversible "modulations" already demonstrated to occur in continuously cultured cell suspensions. It should, however, be recognised that no really critical examination has been made of how far the potential for such "modulation" can be altered by conditions operating during a previous meristematic phase. This should prompt the use of two stage open continuous culture systems: in the first stage the cells would be grown to steady state conditions in different turbidostat environments and then the culture switched to a reduced growth rate in a fixed chemostat environment (with chosen limiting nutrient, precursor supply, physical conditions, and growth factor supply) and the steady states examined in relation to the chosen aspect of cell metabolism.

The possibility of achieving in continuous cultures, cells corresponding to particular differentiated tissue cells of the chosen species must be regarded as a further, and probably more difficult to achieve, objective. A model system for studying cytodifferentiation in plant tissue and cell cultures is presented by photosynthetic parenchyma (mesophyll). In consequence, study of the greening of illuminated cultured tissues and cells has attracted the attention of a number of workers interested in chloroplast reproduction and development and in the development of tissue cultures capable of autotrophic growth. It would be inappropriate to attempt here any comprehensive and critical review of this diverse field of researches. The discussion presented below is based upon work up to 1970 as reviewed by LAETSCH (1971) and on the study of a number of more recent papers to which specific reference is made.

Many of the studies on greening have been related to callus cultures and whenever fine cell suspension cultures have been established in serial culture from such calli, the levels of chlorophyll (per cell and per unit dry weight) have declined. Cellular aggregation and particularly the emergence of organised meristems are clearly promotive of the differentiation of photosynthetically active cells presumably by the establishment of inductive micro-environments within the tissue. It has also been general experience that there is antagonism between growth by cell division and greening (in terms of chlorophyll content, and number and degree of differentiation of chloroplasts). In many instances the effects of phytohormones (NAA and 2,4–D as auxins, kinetin and benzyladenine as cytokinins, and gibberellic acid) have been capable of interpretation in terms of their effects on culture growth. With cell suspensions it has been observed that maximum rise in chlorophyll per unit of culture and particularly per cell occurs as cells enter stationary phase and can continue after growth has ceased (DAVEY *et al.*, 1971). In finely dispersed suspension cultures all or almost all the cells have a similar growth rate, whereas callus cultures always consist of a mosaic of meristematic regions and regions of expanded non-dividing cells (where the antagonism between growth and greening would not operate).

In general green calli are independent of organic growth factors such as vitamins and sugar-alcohols and may be less dependent or independent of phytohormones. The greening of calli is influenced by light intensity and quality, by the composition of the inorganic salt supply (YOSHIDA *et al.*, 1970; YOSHIDA and WATANABE, 1971; YOSHIDA *et al.*, 1973), by the form of nitrogen supply (YOSHIDA and SEKIGUCHI, 1972), by the nature and concentration of phytohormones (e.g. in a number of cases better greening has been reported with NAA than with 2,4-D as auxin—BERGMANN, 1968; DAVEY *et al.*, 1971), and by the supply of sugar (high levels of sugar reducing greening). With some cultures sucrose has seemed to be specifically inhibitory to greening (EDELMAN and HANSON, 1972).

The highest levels of chloroplasts per cell and of chlorophyll per unit dry weight yet achieved in callus cultures are well below those occurring in mesophyll cells of the same species. This is emphasized in such favourable species for greening in culture as *Nicotiana tabacum*. Nevertheless calli of *Ruta graveolens* (CORDUAN, 1970), carrot (HANSON and EDELMAN, 1972) and tobacco (TANDEAU DE-MARSAC and PÉAUD-LENOËL, 1972a, b) and suspension cultures of tobacco (BERGMANN, 1968; CHANDLER *et al.*, 1972) have been obtained which are capable of positive net photosynthesis in presence of enhanced levels of CO_2 (1–2%) and of limited growth (increase in cell dry weight) in the complete absence of sugar.

This is a very promising situation but it is important to consider a number of important reservations which illustrate the unsatisfactory nature of our present ability to closely mimic this pathway of cytodifferentiation in culture: (1) the greening of cultures on exposure to light is extremely slow compared to the response of etiolated organs, (2) chloroplast differentiation in cultured cells is not well synchronised and the same cell may contain chloroplasts of normal fine structure along with plastids whose differentiation is highly abnormal (DAVEY *et al.*, 1971), (3) many cultures completely fail to green or green only very slightly by manipulation of the known variables which will achieve a much higher level of greening in other cultures (LAETSCH, 1971). Considerable variation of greening within the same culture line may be encountered on different occasions, and different laboratories working at least with the same species have achieved very different levels of greening, (4) it is only with tobacco that an autotrophic cell suspension has been obtained; tobacco suspensions normally show considerable cell aggregation. Such reservations as these justify the statement made as recently as 1974 by MARETZKI *et al.* that "conditions for sustained and high photosynthetic rates in cell suspensions still need to be found."

As so often happens in the field of plant tissue and cell culture, we have the tantalising situation that what can be achieved with cultures of a particular cell line or a particular species cannot be extended. Here the situation is also that even with the most favourable cell lines progress has only gone part of the way to reproducing in vitro normal cytodifferentiation. It would seem that not all of the critical factors which impinge on the differentiation of mesophyll cells have yet been identified. If there are such unappreciated factors we would expect inconsistency in results within and between laboratories.

Recent work in our laboratory briefly reported upon at the 3rd International Congress of Plant Tissue and Cell Culture (DALTON, 1974) has shown using cell suspension cultures of *Atropa belladonna* and *Spinacea oleracea* that greening will

only occur to a significant extent in the presence of an appropriate culture atmosphere. The ethylene produced by the cultures is antagonistic to greening; its effect can be antagonised by enhancing the level of carbon dioxide in the culture atmosphere. Quite high levels of CO_2 (5–10%) are needed to antagonise the levels of ethylene (5–20 ppm.) which can readily build up in shake cultures. The relatively high levels of CO_2 utilised in experiments on autotrophic growth may well be therefore, in excess of the CO_2 levels necessary to saturate the CO_2–fixation reaction but nevertheless be beneficial because of their antagonising an ethylene effect. Experiments in which the level of ethylene in the gas phase of the cultures have been maintained at less than 1.0 ppm. by use of an ethylene absorbant have revealed the additional importance to greening of a low partial pressure of oxygen. The most effective conditions of oxygen supply involve a level of oxygen below the air level (5% O_2 + 95% N_2) flowing through the culture at a rate adequate to allow fully aerobic respiration. This suggests that an appropriate intracellular oxidation-reduction potential is critical to greening. The concept of the importance of the gas phase in cell culture is also supported by studies on the effects of CO_2 tensions in relation to the growth of cells at very low densities (MANSFIELD via STREET, 1973a, GATHECOLE *et al.*, 1976) and in relation to cell permeability (DORÉE *et al.*, 1972), and by studies on the influence of dissolved oxygen concentration on morphogenetic expression in carrot cultures (KESSEL and CARR, 1972).

It is now urgently necessary to see how far such "gaseous factors" are the previously unappreciated factors in mesophyll differentiation in culture. If this should prove to be the case further study of the greening process may not only provide valuable and reproducible material for studies on chloroplast differentiation and photosynthesis, but provide us with a model approach for the study of other aspects of cytodifferentiation in culture.

6. Genetic Stability of Cell Suspension Cultures —Implications for Their Application

The cytological instability characteristic of plant tissue and cell cultures has been reviewed recently by SUNDERLAND (1973a). More recent work by BAYLISS (1973, 1975) with various cell suspensions has extended our knowledge of the mechanisms involved and of how they are affected by cultural factors. The highly stable cell lines which can arise in culture are lines of altered genome. Further they may at any time precipitately undergo cytological breakdown and gradually give rise to a further but different line which is relatively stable. Even under the most favourable conditions of culture, cytological deviations are constantly arising and are subjected to selection pressure in the culture environment. The culture environment of the growing cell suspension selects for capacity to divide and grow; it does not select for conformity to the other factors essential to continuing survival and/or multiplication within the organism (capacity to integrate within the organisation of tissues and organs). Further, any cytological variant which has

any selective advantage (reduced lag phase, enhanced capacity for nutrient utilisation, enhanced survival during stationary phase) within the cell suspension will come to be the dominant cell type. Suspension cultures normally, therefore, undergo cytological drift exhibiting periods of cytological heterogeneity where there exist mixed populations due to survival and further change in cytological deviants. These periods alternate with periods (which may be extended) where the culture has a single or very dominant chromosome mode; the very low frequency of deviants often recorded in such cultures possibly represents cells which are continuously being eliminated by subculture.

The immediate conclusion which follows from this situation is that any cell line selected for its high secondary product synthesis will need to be preserved by cryobiological methods (see Chap. VII.3 of this Vol.). The cytological instability of cell suspension cultures also points to the continuous production of genetic variation which might be exploited to achieve higher production of a selected plant product. The ability to select anti-metabolite resistant cell lines from established cultures (BINDING, 1974; WIDHOLM, 1974) illustrates that this variation throws up cell lines differing in metabolite production, in permeability properties, and in the sensitivity of their enzymes to inhibition. In this connection it should be pointed out that the 5 l continuous culture systems described above may contain at least 10^{10} cells and that by appropriate alteration of the composition of the input medium (e.g. omission of organic nutrients, growth factors, or phytohormones; inclusion of anti-metabolites or enzyme inhibitors; alteration of medium pH, culture temperature, gas atmosphere) the population can be continuously subjected to selection pressure. This may prove particularly valuable for the selection of rare variants having desired qualities.

The possibility of obtaining recessive mutations is now opened up by the availability of haploid cell lines from a limited number of genera (SUNDERLAND, 1973b; Chap. II.1 of this Vol.). Work is currently proceeding with cell suspensions rich in haploid cells (RASHID and STREET, 1974) to isolate biochemical mutants following treatment with mutagens such as ethyl methane sulphonate (EMS) (STREET, 1975) and single cell cloning (STREET, 1973a; Chap. V.1 of this Vol.). Such work is currently hampered by lack of appropriate selection techniques but, as indicated earlier, it may be possible to recognize secondary product mutant colonies where the products can be detected by tests which do not destroy cell viability. If the required mutant is an auxotroph it may be possible to develop the 5-bromodeoxyuridine (BudR) technique for the selective kill of "wild" type cells and thereby generally enhance the frequency of the desired class of mutant in the surviving population. However, in our experience, the BudR technique as described by CARLSON (1970) is not of wide application. In this connection the possibility should be explored of obtaining a thymidine-minus parent strain or of using A-methopterin to exhaust the thymidine pool in growing wild type cells (DEMARS and HOOPER, 1960).

The possibility of isolating mutants in which regulatory genes are inactivated has been suggested as an alternative to working out empirically the exact sequential treatment which may be required to achieve in suspension cultures a specific pathway of cytodifferentiation (STREET, 1973c). This approach may prove valid whether cytodifferentiation involves changes at the transcriptional or transla-

tional level of gene expression or a combination of these. What is advocated here is that the mutational approach should *not* be restricted to those species and cell lines which already produce secondary products without having to embark upon an established pathway of cytodifferentiation.

The next and most exciting phase in the application of cell suspension cultures as biosynthetic systems should develop from the use of selected mutant cell lines tested as steady state systems under a wide range of cultural environments, as is now possible with the recently validated techniques of closed and open continuous culture.

References see page 703.

2. Secondary Products in Tissue Cultures

D. N. BUTCHER

1. Introduction

Plant tissue cultures are potentially valuable for studying the biosynthesis of secondary metabolites and may also eventually provide an efficient means of producing commercially important plant products. The aim of this article is to assess the progress that has been made towards the realisation of these potentialities for compounds other than those which have medical importance. The latter compounds are considered in Chapter VI.3 of this Volume. Here the terms secondary product and secondary metabolite will be confined to those compounds of secondary metabolism which are not thought to be essential to all plant cells. This definition includes many compounds for which no definite role has been assigned, and also compounds such as lignins and flower pigments which have known functions. There will be no attempt to give a comprehensive treatment of the subject, but rather aspects will be selected to illustrate the advantages and disadvantages of employing culture techniques to studies of secondary product biosynthesis. Recent articles on this subject include those of KRIKORIAN and STEWARD (1969), PUHAN and MARTIN (1971), STREET (1973) and CONSTABEL *et al.* (1974).

2. The Suitability of Tissue Cultures for Biosynthetic Studies

On first examination it would appear that cultured cells offer many advantages over intact plants for biosynthetic studies. They are relatively easy to grow and can be kept under strictly controlled nutritional and environmental conditions. Hence the uncertainties of climate and soils can be avoided. They are also cultured aseptically which eliminates the problems associated with contaminations by microorganisms. Further, cultures often provide simpler and more convenient experimental systems than intact plants. In particular, cultures grown in liquid media offer a very effective way of incorporating precursor materials which are often difficult to administer to the entire plant. Finally, and perhaps the most attractive feature, is that the technology is now available for the relatively large-scale production of plant cell suspensions in batch cultures, chemostats and turbidostats (see Chap. VI.1 of this Vol.).

In spite of these obviously desirable features the use of tissue cultures has so far been very limited, mainly because many cultures do not produce significant amounts of the compounds characteristic of the plant from which they were

derived. This inability may sometimes be due to a loss of genetic information during prolonged culture, but this seems unlikely in the many cases where it has been shown that the cells are totipotent. It is more probable that the failure to produce secondary metabolites is a consequence of the special physiological and morphological properties of cultured tissues. Except in the case of organ cultures, they are by and large composed of disorganised tissues and possess a very limited range of cell types. Indeed tissue culture techniques and procedures have been designed to keep cells in the dividing state, which tends to limit cell differentiation and development of organised tissue systems. The extreme examples are seen with "ideal" suspension cultures which are composed of single cells and small aggregates of parenchyma-like cells. Since the synthesis of many secondary products is associated either with specialised differentiated cell types or with organised tissue systems, it is perhaps not surprising that some secondary metabolites are not produced in culture. As we shall see, one of the major factors which determines whether or not a particular metabolite is accumulated is the closeness of the relationship between the production of the compound and differentiated structures in the intact plant. Other factors, such as light, temperature, plant hormones and availability of precursors, which are known to influence the production of secondary metabolites in intact plants, also have to be taken into account, but usually these do not present insuperable problems. In the following sections the various classes of secondary products will be considered in groups in accordance with their biochemical origins. In addition, a list of the compounds which have been detected in tissue cultures is given in Table 1.

3. Cinnamic Acids, Coumarins and Lignins

3.1 Cinnamic Acids and Their Simple Derivatives

Although there have been relatively few reports of the isolation of free cinnamic acids from tissue cultures, free and bound forms of cinnamic, caffeic and ferulic acids have been detected in tobacco callus (BROWN and TENNISWOOD, 1974). Callus cultures of tobacco have also been found to contain p-coumaroyl putrescine, caffeoyl putrescine and ferulo-putrescine, compounds which are not present in the intact plant (MIZUSAKI *et al.*, 1971). In addition, chlorogenic acid, a derivative of caffeic acid, has been isolated from callus cultures of tobacco (SARGENT and SKOOG, 1960); and *Haplopappus gracilis* (STICKLAND and SUNDERLAND, 1972a); and suspension cultures of potato (GAMBORG, 1967).

The accumulation of cinnamic acids and their derivatives has been found to be influenced by the cultural conditions. High levels of auxin caused a marked decrease in the amounts of p-coumaric and ferulic acids present in flax callus with a concomitant increase in the amounts of p-hydroxybenzoic and vanillic acids, probable oxidation products of the cinnamic acids. In contrast, high levels of kinetin led to increased amounts of p-coumaric and ferulic acids. Similarly the addition of phenylalanine, a likely precursor, also resulted in higher levels of cinnamic acids (LIAU and IBRAHIM, 1973). The production of chlorogenic acid in

Table 1. Secondary products detected in plant tissue cultures

Compound	Plant source	Type of culture	Reference
Cinnamic acids and their derivatives			
Cinnamic acid	*Nicotiana tabacum*	C	BROWN and TENNISWOOD (1974)
Caffeic acid	*N. tabacum*	C	BROWN and TENNISWOOD (1974)
Ferulic acid	*N. tabacum*	C	BROWN and TENNISWOOD (1974)
Ferulic acid	*Linum usitatissimum*	C	LIAU and IBRAHIM (1973)
p-coumaric acid	*L. usitatissimum*	C	LIAU and IBRAHIM (1973)
Caffeoyl putrescine	*N. tabacum*	C	MIZUSAKI *et al.* (1971)
Ferulo putrescine	*N. tabacum*	C	MIZUSAKI *et al.* (1971)
p-coumaroyl putrescine	*N. tabacum*	C	MIZUSAKI *et al.* (1971)
Chlorogenic acid	*Haplopappus gracilis*	C	STICKLAND and SUNDERLAND (1972a)
Chlorogenic acid	*Solanum tuberosum*	S	GAMBORG (1967)
Benzoic acids			
p.-hydroxybenzoic acid	*L. usitatissimum*	C	LIAU and IBRAHIM (1973)
Vanillic acid	*L. usitatissimum*	C	LIAU and IBRAHIM (1973)
Coumarins			
Scopoletin	*N. tabacum*	C	SARGENT and SKOOG (1960, 1961)
Scopoletin	*N. tabacum*	C and CGC	BROWN and TENNISWOOD (1974)
Scopoletin	*Ruta graveolens*	S	REINHARD *et al.* (1968); STECK *et al.* (1971)
Esculetin	*N. tabacum*	C and CGC	BROWN and TENNISWOOD (1974)
Umbelliferone	*N. tabacum*	C	BROWN and TENNISWOOD (1974)
Umbelliferone	*R. graveolens*	S	REINHARD *et al.* (1968); STECK *et al.* (1971)
Bergapten	*N. tabacum*	C	BROWN and TENNISWOOD (1974)
Bergapten	*R. graveolens*	S	REINHARD *et al.* (1968)
Psoralen	*R. graveolens*	S	REINHARD *et al.* (1968); STECK *et al.* (1971)
Xanthotoxin	*R. graveolens*	S	REINHARD *et al.* (1968); STECK *et al.* (1971)
Herniarin	*R. graveolens*	S	REINHARD *et al.* (1968)
Rutaretin	*R. graveolens*	S	REINHARD *et al.* (1968)
3-(1,1-dimethylallyl)-scopoletin	*R. graveolens*	S	BROCKE *et al.* (1971)
Isopimpinellin	*R. graveolens*	S	STECK *et al.* (1971)
Rutacultin	*R. graveolens*	S	STECK *et al.* (1971)
Rutamarin	*R. graveolens*	S	STECK *et al.* (1971)
Flavones and flavonols			
Apigenin	*Petroselinum hortense*	S	KREUZALER and HAHLBROCK (1973); HAHLBROCK and WELLMANN (1970)
Apigenin	*Glycine max*	S	HAHLBROCK (1972)
Luteolin	*P. hortense*	S	KREUZALER and HAHLBROCK (1973)
Chrysoeriol	*P. hortense*	S	KREUZALER and HAHLBROCK (1973)
Sinensetin	*Citrus aurantium*	C	BRUNET and IBRAHIM (1973)
Nobiletin	*C. aurantium*	C	BRUNET and IBRAHIM (1973)
Isorhamnetin	*P. hortense*	S	KREUZALER and HAHLBROCK (1973)
Quercetin	*P. hortense*	S	KREUZALER and HAHLBROCK (1973)
Quercetin	*Crotalaria juncea*	C	JAIN and KHANNA (1974)
Chalcones and deoxyflavones			
2′,4,4′-trihydroxychalcone	*Phaseolus aureus*	C	BERLIN and BARZ (1971)
Daidzein	*P. aureus*	C and S	BERLIN and BARZ (1971)
Daidzein	*G. max*	C	MILLER (1969)

Table 1 (continued)

Compound	Plant source	Type of culture	Reference
Coumestanes and coumarinochromans			
Coumestrol	*P. aureus*	C and S	BERLIN and BARZ (1971)
Soyagol	*P. aureus*	C and S	BERLIN and BARZ (1971)
Pisatin	*Pisum sativum*	C	BAILEY (1970)
Anthocyanins			
Cyanidin	*H. gracilis*	C	ARDENNE (1965); STICKLAND and SUNDERLAND (1972a)
Cyanidin	*L. usitatissimum*	C	IBRAHIM *et al.* (1971)
Cyanidin	*Dimorphotheca auriculata*	C	HARBORNE *et al.* (1970)
Delphinidin	*D. auriculata*	C	HARBORNE *et al.* (1970)
Tannins and tannin precursors			
Catechin	*Camellia sinensis*	C	FORREST (1969)
Catechin	Paul's Scarlet Rose	S	DAVIES (1972a)
Epicatechin	*C. sinensis*	C	FORREST (1969)
Epicatechin	Paul's Scarlet Rose	S	DAVIES (1972a)
D-glucogallin	Paul's Scarlet Rose	S	DAVIES (1972a)
Leucoanthocyanins	*C. sinensis*	C	FORREST (1969)
Anthraquinones			
Digitolutein	*Digitalis lanata*	C	FURUYA and KOJIMA (1971)
4-hydroxydigitolutein	*D. latana*	C	FURUYA and KOJIMA (1971)
3-methylpurpurin	*D. latana*	C	FURUYA *et al.* (1972)
3-methylquinizarin	*D. latana*	C	FURUYA *et al.* (1972)
3-methylalizarin	*D. latana*	C	FURUYA *et al.* (1972)
Pachybasin	*D. latana*	C	FURUYA *et al.* (1972)
Morindone	*Morinda citrifolia*	S	LEISTNER (1973)
Alizarin	*M. citrifolia*	S	LEISTNER (1973)
damnacanthal	*M. citrifolia*	S	LEISTNER (1973)
Chrysophanol, Physcion, Rheum emodin	*Cassia angustifolia*	C	FRIEDRICH and BAIER (1973)
	Cassia tora	C	TABATA *et al.* (1975)
Aloe emodin	*C. angustifolia*	C	FRIEDRICH and BAIER (1973)
Rhein	*C. angustifolia*	C	FRIEDRICH and BAIER (1973)
Naphthoquinones			
Plumbagin	*Plumbago zeylanica*	C	HEBLE *et al.* (1974)
Sesquiterpenes			
Lindenenol	*Lindera strychnifolia*	C	TOMITA *et al.* (1969)
Lindenenol acetate	*L. strychnifolia*	C	TOMITA *et al.* (1969)
Linderane	*L. strychnifolia*	C	TOMITA *et al.* (1969)
Linderalacton	*L. strychnifolia*	C	TOMITA *et al.* (1969)
Lindesterene	*L. strychnifolia*	C	TOMITA *et al.* (1969)
Caryophyllene	*L. strychnifolia*	C	TOMITA *et al.* (1969)
Paniculides A, B and C	*Andrographis paniculata*	C and S	ALLISON *et al.* (1968); BUTCHER and CONNOLLY (1971)
Trans, trans and *cis, trans* farnesols	*A. paniculata*	S	OVERTON and ROBERTS (1974 a, b)
γ-bisabolene	*A. paniculata*	S	OVERTON and ROBERTS (1974 a, b)
Sterols and triterpenes			
β-sitosterol, Stigmasterol, Campesterol	*N. tabacum*	C	BENVENISTE *et al.* (1966)
	Dioscorea tokoro	C	TOMITA *et al.* (1970)
	Withania somnifera	C	YU *et al.* (1974)
	Paul's Scarlet Rose	C	WILLIAMS and GOODWIN (1965)
	Tylophora indica	C	BENJAMIN and MULCHANDANI (1973)
	Helianthus annuus	C	BUTCHER *et al.* (1974)

Table 1 (continued)

Compound	Plant source	Type of culture	Reference
Cholesterol	*H. annuus*	CandCGC	BUTCHER *et al.* (1974)
Isofucosterol	*H. annuus*	CandCGC	BUTCHER *et al.* (1974)
Cycloartenol	*N. tabacum*	C	BENVENISTE *et al.* (1966); BENVENISTE (1968)
2,4-methylene cycloartenol	*N. tabacum*	C	BENVENISTE *et al.* (1966); BENVENISTE (1968)
Citrostradienol	*N. tabacum*	C	BENVENISTE *et al.* (1966); BENVENISTE (1968)
Citrostradiol	*N. tabacum*	C	BENVENISTE *et al.* (1966); BENVENISTE (1968)
Cycloeucalenol	*N. tabacum*	C	BENVENISTE *et al.* (1966); BENVENISTE (1968)
Obtusifoliol	*N. tabacum*	C	BENVENISTE *et al.* (1966); BENVENISTE (1968)
2,4-ethylidene cholesterol	*W. somnifera*	C	YU *et al.* (1974)
2,4-methylene cholesterol	*W. somnifera*	C	YU *et al.* (1974)
β-amyrin	Paul's Scarlet Rose	C	WILLIAMS and GOODWIN (1965)
β-amyrin	*T. indica*	C	BENJAMIN and MULCHANDANI (1973)
Arundoin	*Oryza sativa*	C	YANAGAWA *et al.* (1972)
Steroidal alkaloids			
Tomatine	*Lycopersicum esculentum*	C	RODDICK and BUTCHER (1972a)
Solasonine	*Solanum xanthocarpum*	C	HEBLE *et al.* (1971)
Carotenoids			
Voilaxanthin, Zeaxanthin, Neoxanthin	Paul's Scarlet Rose	*C*	WILLIAMS and GOODWIN (1965)
	R. graveolens	*C*	SCHARLEMANN and CZYGAN (1971)
Auroxanthin	Paul's Scarlet Rose	*C*	WILLIAMS and GOODWIN (1965)
β-carotene	*R. graveolens*	*C*	SCHARLEMANN and CZYGAN (1971)
Lutein	*R. graveolens*	*C*	SCHARLEMANN and CZYGAN (1971)
Lutein-5,6 epoxide	*R. graveolens*	*C*	SCHARLEMANN and CZYGAN (1971)
Antheraxanthin	*R. graveolens*	*C*	SCHARLEMANN and CZYGAN (1971)
Unusual fatty acids and related compounds			
Sterculic acid, Dihydrosterculic acid, Malvalic acid, Dihydromalvalic acid	*Malva paviflora*	C	YANO *et al.* (1972a)
	Malva sylvestris	C	YANO *et al.* (1972a)
Hydnocarpic acid	*Hydnocarpus anthelmintheca*	C	SPENER *et al.* (1974)
Chaulmoogric acid	*H. anthelmintheca*	C	SPENER *et al.* (1974)
Gorlic acid	*H. anthelmintheca*	C	SPENER *et al.* (1974)
Erucic acid	*Crambe abyssinica*	C	JONES (1974)
2-undecanone	*R. graveolens*	C	REINHARD *et al.* (1971); CORDUAN and REINHARD (1972)
2-undecanyl acetate	*R. graveolens*	C	REINHARD *et al.* (1971); CORDUAN and REINHARD (1972)
2-nonanone	*R. graveolens*	C	REINHARD *et al.* (1971); CORDUAN and REINHARD (1972)
2-nonanyl acetate	*R. graveolens*	C	REINHARD *et al.* (1971); CORDUAN and REINHARD (1972)
2-nonanol	*R. graveolens*	C	REINHARD *et al.* (1971); CORDUAN and REINHARD (1972)
2-undecanol	*R. graveolens*	C	REINHARD *et al.* (1971); CORDUAN and REINHARD (1972)
Aliphatic alkanes C_{17}–C_{28}	*N. tabacum*	C	WEETE *et al.* (1971)

Table 1 (continued)

Compound	Plant source	Type of culture	Reference
Glucosinolates			
Benzylglucosinolate	*Tropeaolum majus*	S	KIRKLAND *et al.* (1971)
2-phenylethyl glucosinolate	*T. majus*	S	KIRKLAND *et al.* (1971)
2-hydroxy-2-phenyl-ethylglucosinolate	*Reseda luteola*	S	KIRKLAND *et al.* (1971)
Glucobrassicin	*Brassica rapa*	C	EL-TIGANI (1972)
Neoglucobrassicin	*B. rapa*	C	EL-TIGANI (1972)

Key: C = callus; CGC = crown-gall callus; S = suspension cultures.

callus of *H. gracilis* was enhanced by white, blue and red light, with blue being the most effective. On the other hand, the addition of 2,4-dichlorophenoxy-acetic acid (2,4-D) at 0.1 mg/l caused a substantial reduction in the quantity of chlorogenic acid produced (STICKLAND and SUNDERLAND, 1972b). It has also been shown with suspension cultures of potato that ^{14}C labelled shikimic acid, quinic acid, p-coumaric acid and caffeic acid can serve as precursors of chlorogenic acid (GAMBORG, 1967).

3.2 Coumarins

Scopoletin and 4 of its glycosides (fabiatin, β-gentibioside, scopolin and an unknown glucoside) have been isolated from callus and intact cortical tissues of tobacco (SARGENT and SKOOG, 1960, 1961). The amounts of scopoletin and scopolin produced were shown to be markedly influenced by the levels of auxin and kinetin and the type of auxin (IAA or 2,4-D) in the medium (SKOOG and MONTALDI, 1961; FURUYA *et al.*, 1971). Furthermore, in a comparative study it has been found that normal tobacco cultures contain scopoletin, esculetin, umbelliferone and the furanocoumarin, bergapten, while crown-gall cultures contain large amounts of free and bound forms of scopoletin and esculetin, but no umbelliferone or bergapten (BROWN and TENNISWOOD, 1974).

Some of the most interesting work on coumarins has been done with callus and suspension cultures derived from *Ruta graveolens*. The coumarins present in these cultures included psoralen, bergapten, xanthotoxin, rutaretin, herniarin, scopoletin, umbelliferone and a new compound 3-(1,1-dimethylallyl)-scopoletin (Fig. 1, REINHARD *et al.*, 1968; BROCKE *et al.*, 1971). The patterns of the coumarins present in the suspension cultures differed from those present in callus, and significant amounts were excreted into the medium. Other factors which influenced the compounds produced included light and type of auxin in the medium. In a subsequent study STECK *et al.* (1971) confirmed the presence of umbelliferone, scopoletin, psoralen and xanthotoxin in suspension cultures of *R. graveolens*, but did not detect herniarin, bergapten or rutaretin. However they reported isopimpi-

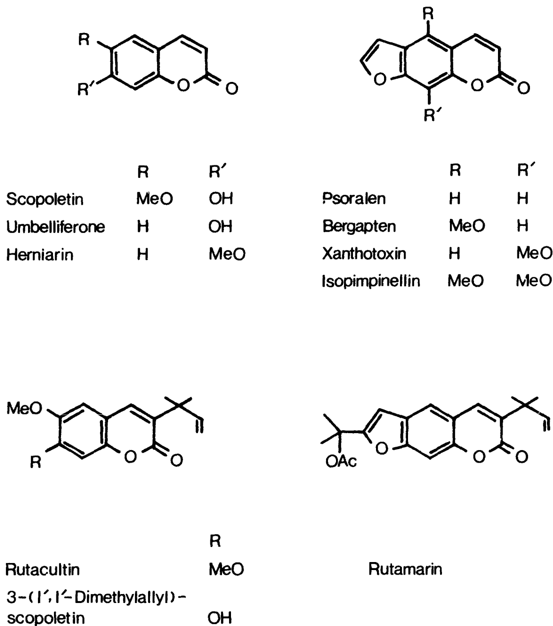


Fig. 1. The coumarins and furanocoumarins in cultures derived from *Ruta graveolens*

nellin, rutamarin and a new compound rutacultin, 6,7-dimethyloxy-3-(1,1-dimethyl) coumarin (Fig. 1). Although several of the coumarins discovered in the suspension cultures were found in intact plants, isopimpinellin and rutacultin were not detected, and rutamarin was present only in trace amounts. In contrast to the previous work the levels of auxins and kinetin in the medium did not appear to influence the yields or patterns of coumarins produced. Further, the presence of shoot-like structures in cultures grown in the absence of auxin had little effect on the coumarins present.

More recently AUSTIN and BROWN (1973) have treated cultures of *R. graveolens* with various ^{14}C labelled compounds in order to study the biosynthetic pathways leading to the furanocoumarins, psoralen, xanthotoxin, bergapten and isopimpinellin. Umbelliferone, 7-dimethylsuberosin and (±)-marmesin were well incorporated compared with mevalonic acid. This supported the view that the former compounds were intermediates in the furanocoumarin pathway. It was concluded that although the biosynthetic pathways were basically similar in tissue cultures and in intact tissues, there were some obvious differences as indicated by differences in the utilization of precursors.

It is clear that tissue cultures often produce significant amounts of coumarins, and may be used effectively in biosynthetic studies. It is perhaps significant that in intact plants coumarins, when present, are frequently distributed throughout the tissues and do not appear to be associated with specialised cell types. In intact tissues the coumarins are often found in the vacuoles, while in tissue culture they are in the vacuoles, but may also be excreted into the medium. It is interesting that cultures of *R. graveolens* produce many of the unusual coumarins found in the intact plant, plus some new compounds.

3.3 Lignins

It is well known that many callus cultures possess lignified cells, and consequently several workers have used such cultures for studying the process of lignification. The lignin most commonly occurs in the cell walls of tracheid-like elements, but it has also been found in the walls of parenchyma-like cells and inter-cellular spaces (VENVERLOO, 1969; CARCELLER *et al.*, 1971).

Lignin isolated from *Pinus strobus* callus cultures was shown to be of the typical guaiacyl-type conifer lignin (HASEGAWA *et al.*, 1960; HIGUCHI *et al.*, 1960). On the other hand, cultures derived from angiosperms such as *Paulownia tormentosa*, *Phaseolus vulgaris*, *Populus nigra* and *Populus tremuloides*, *Solanum tuberosum* did not contain lignin of typical angiosperm wood (HIGUCHI *et al.*, 1960; GAMBORG, 1967; JEFFS and NORTHCOTE, 1967; VENVERLOO, 1969; WOLTER *et al.*, 1974). The lignin in these cultures had low syringyl: guaiacyl ratios resembling conifer wood, and also low numbers of methoxyl groups.

Biosynthetic studies with ^{14}C labelled compounds showed that shikimic acid, phenylalanine, p-coumaric acid and ferulic acid were good precursors for lignin biosynthesis in *Pinus strobus* (HASEGAWA *et al.*, 1960). Similarly ^{14}C labelled cinnamic, p-coumaric and caffeic acids were incorporated into lignin of suspension cultures of potato (GAMBORG, 1967). In addition BARNOUD (1965) found that coniferin and syringin, probable precursors of lignin synthesis, when added to the medium, enhanced lignin synthesis while reducing the growth rate in cultures of *Syringa vulgaris*.

Both auxins and cytokinins have been found to affect lignin formation in callus cultures. Kinetin, when added at the appropriate concentration, enhanced the amount of lignin in callus of *Daucus carota* (KOBLITZ, 1962) and *Nicotiana tabacum* (BERGMANN, 1964). On the other hand, auxin and sucrose levels in the medium of callus cultures of *Syringa vulgaris* and *Phaseolus vulgaris* greatly influenced the formation of xylem and degree of lignification (WETMORE and RIER, 1963; JEFFS and NORTHCOTE, 1967). Furthermore the amount and type of lignin in callus cultures of *Populus nigra* depended very much on the nature of the auxin present in the medium (VENVERLOO, 1969). In recently initiated cultures the syringyl: vanillin ratio was lower in callus grown on 2,4-D medium than in cultures grown on IAA medium. Moreover the 2,4-D cultures lost their ability to form lignified tracheids after about three years while the IAA cultures retained this ability.

In all the above studies it is difficult to decide whether the effects of the growth hormones are directly on lignification or on vascular differentiation. However, CARCELLER *et al.* (1971) have shown that the lignin contents of sycamore suspension cells, in which no tracheids are formed, are very much affected by the concentrations of sucrose, 2,4-D and kinetin in the medium. This indicates that the effects of these substances on lignin biosynthesis, in this case at least, are not dependent on the differentiation of xylem-like elements.

Several other factors have been shown to affect lignin biosynthesis in tissue cultures. Light stimulates lignin formation in callus of *Pinus strobus*, which is not surprising since the activity of phenylalanine-ammonia-lyase (PAL), a key enzyme in the phenylpropanoid pathway, is known to be influenced by light. Indeed a

correlation between lignin formation in vascular nodules and PAL activity has been observed in newly established callus from hypocotyls of *Phaseolus vulgaris* (HADDON and NORTHCOTE, 1975). In addition, LIPETZ (1962) has shown that low levels of calcium in the medium enhance lignin synthesis in callus cultures of *Parthenocissus tricuspidata*, *N. tabacum* and *D. carota*.

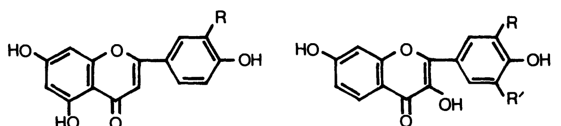
The investigations so far suggest that valuable information regarding lignin biosynthesis may be gained from studies with tissue cultures, but the discovery that the composition of lignins in tissue cultures is often different from that of the intact plant has to be taken into account when relating the results to lignin biosynthesis in intact plants.

4. Flavonoids

4.1 Flavones, Flavonols, Chalcones, and Isoflavones

These classes of flavonoids have a widespread distribution in intact plants, and have been found in a number of tissue cultures. The most detailed work has been done on suspension cultures of *Petroselinum hortense*, which produced 24 glycosides of flavones and flavonols when grown in the light (HAHLBROCK and WELLMANN, 1970; KREUZALER and HAHLBROCK, 1973). The aglycones identified included the flavones, apigenin, luteolin and chrysoeriol, and the flavonols, quercetin and isorhamnetin (Fig. 2). The flavones occurred either as 7-O-glucosides or as 7-O-apioglucosides, while the flavonols were either 3-O-monoglucosides or 3-7-O-diglucosides. Some of the glycosides were substituted with malonyl residues. The presence of the glucosides was dependent on light and their formation began after a lag phase of 4–6 h, and reached a constant level after 3–4 days. WELLMANN (1971) found that irradiations with wavelengths below 320 nm (UV) were very effective in stimulating flavone synthesis, and that little synthesis occurred when the cultures were exposed to the visible wavelengths alone. However, after a pre-irradiation with UV light the involvement of the red/far red phytochrome system has been demonstrated (WELLMANN and SCHOPFER, 1975).

Many of the enzymes concerned with flavonoid biosynthesis in suspension cultures of *P. hortense* have been detected and partially characterised (HAHLBROCK *et al.*, 1971; SUTTER *et al.*, 1972; ORTMANN *et al.*, 1972). It was observed that although dark-grown cell cultures did not form flavonoid pigments, many of the enzymes involved in flavone glycoside biosynthesis were detectable. While the activities of most of these enzymes were very low, those of cinnamic acid-4-hydroxylase and p-coumarate:CoA ligase represented 30–40% of the values in illuminated cells. It was concluded that the activities of these enzymes were not limiting flavone biosynthesis in the dark in spite of the observation that there was always an increase in their activities when the cultures were illuminated. When dark-grown cultures were exposed to continuous light the increases in the activities of PAL, cinnamic acid-4-hydroxylase and p-coumarate:CoA ligase reached maxima after approximately 15 h, while the remaining enzymes of the pathway to apiin showed maximum activities after 24 h. It was suggested that the first group



	R
Apigenin	H
Luteolin	OH
Chrysoeriol	OMe

	R	R′
Quercetin	OH	H
Isorhamnetin	Me	H

Fig. 2. Flavones and flavonols found in suspension cultures of *Petroselinum hortense*

of enzymes, which were involved in the synthesis of other phenylpropanoids besides the flavonoids, were regulated by a different mechanism from those which were specific to flavonoid biosynthesis. In support of this view HAHLBROCK and RAGG (1975) found that the light-induced activities of the first group of enzymes were inhibited by low concentrations of actinomycin D and cycloheximide, while those of the other group were unaffected. Other investigations have indicated that the light-induced increases in PAL activity were due to *de novo* synthesis and not to an activation of pre-formed inactive enzyme (HAHLBROCK and SCHRÖDER, 1975). However the interpretation of all these experiments has been handicapped by the fact that enzyme activities changed during the culture period even during darkness, and that the amounts of activity depended very much on the size of the inoculum. Furthermore, it has been shown that light-induced PAL activity varies considerably according to the age of the culture (ZIMMERMANN and HAHLBROCK, 1975).

The flavone, apigenin, has also been isolated from illuminated suspension cultures of *Glycine max*, a plant which is known to produce aurones, chalcones and isoflavones. In this case the major part of the flavone accumulation was confined to the end of the growth period (HAHLBROCK, 1972). A correlation between apigenin biosynthesis and PAL and p-coumarate:CoA ligase activities was also observed.

Two glycosides of the deoxyisoflavone, daidzein, have been isolated from callus cultures of *G. max* when grown on media containing auxin and cytokinin (MILLER, 1969). These compounds could be detected in the cultures after a lag period of 24 h. Different auxins and various concentrations of auxins and cytokinins in the medium, influenced the production of daidzein, but these effects seemed to correspond to the effects of these treatments on growth.

Callus cultures and cell suspensions of *Phaseolus aureus* have been shown to produce daidzein, the coumestanes, coumestrol and soyagol, and 2′,4,4′-trihydroxychalcone (Fig. 3) (BERLIN and BARZ, 1971). When cultured for prolonged periods in liquid media, callus masses were gradually replaced by a dispersed cell suspension, and this was accompanied by a decrease in the amount of flavonoids produced. It is possible that this decrease was due to the presence of degradative enzymes since it has been shown that these suspension cultures can metabolise daidzein and other phenylpropanoid compounds.

Callus cultures originating from orange flavedo produced significant amounts of sinensetin, nobiletin and other flavones when exposed to light, but not in

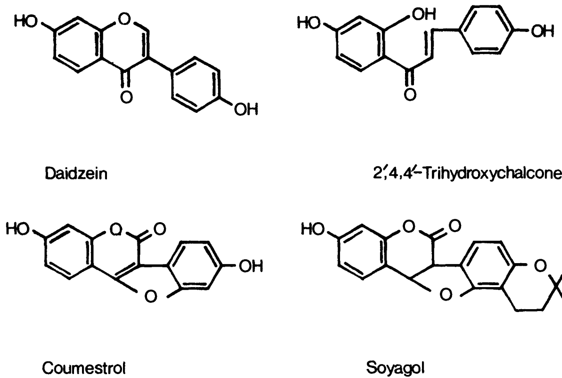


Fig. 3. Flavonoids isolated from cultures of *Phaseolus aureus*

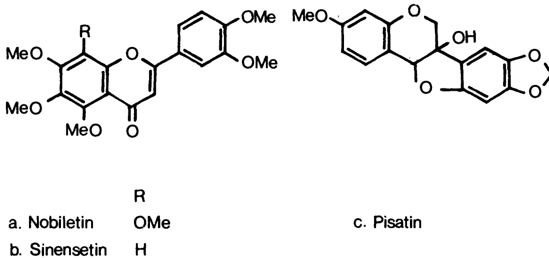


Fig. 4a–c. Flavonoids found in callus derived from orange flavedo (a and b) and *Pisum sativum* (c)

darkness (Fig. 4), (BRUNET and IBRAHIM, 1973). However the total flavonoid contents were very low compared with peel explants. Again a rapid increase in PAL activity preceded the production of these flavonoids. The presence of the coumarinochroman, pisatin, a phytoalexin, has been demonstrated in uninfected callus cultures of *Pisum sativum* (Fig. 4) (BAILEY, 1970). Pisatin production appeared to depend on the presence of coconut milk in the medium, and the highest amounts accumulated in slow-growing pigmented cultures. After prolonged periods in culture and the selection of fast-growing calli, the capacity of the cultures to accumulate pisatin declined.

Although many flavonoids do occur in tissue cultures, the types and quantities produced are often restricted compared with the intact plants. In some cases this could be due to the presence in cultures of degradative enzymes. This possibility is supported by the finding that cell suspension cultures of *Cicer arietinum*, *Petroselinum hortense*, *Phaseolus aureus* and *G. max* can metabolise ^{14}C labelled kaempferol, quercetin, isorhamnetin and 4,6-dihydroxyaurone (HÖSEL *et al.*, 1972; BARZ *et al.*, 1974).

4.2 Anthocyanins

The anthocyanins occur in a large number of callus and suspension tissues. The pigment-containing cells may be distributed diffusely or may be localised, giving a patchy appearance to the cultures. It is interesting that in several cases it has been possible to isolate highly pigmented sub-strains which accumulate massive

amounts of anthocyanins. The purple or blue strains of *Haplopappus gracilis* (REINERT *et al.*, 1964; STICKLAND and SUNDERLAND, 1972a), and *Daucus carota* (ALFERMANN and REINHARD, 1971) are good examples of this, and have provided excellent material for studies of the factors which influence anthocyanin biosynthesis.

ARDENNE (1965) identified the anthocyanins present in a highly pigmented strain of *H. gracilis* as cyanidin-3-glucoside and cyanidin-3,5-diglucoside. On the other hand STICKLAND and SUNDERLAND (1972a) found cyanidin-3-glucoside and cyanidin-3-rutinoside in another purple strain derived from the same species. Glycosides of cyanidin, malvidin and delphinidin have been isolated from callus cultures of several other species, and it has been shown that the pigments produced are often different from those accumulated by the intact plant. For instance cultures of *Dimorphotheca auriculata* produced cyanidin-3-glucoside and delphinidin-3-glucoside, compounds not found in the intact stems when cultured on media containing 2,4-D (HARBORNE *et al.*, 1970). Furthermore callus cultures derived from *Linum usitatissimum* produced only cyanidin-3,5-diglucoside and not the highly methylated malvidin and hirsutidin glycosides which accumulate in the hypocotyls and petals (IBRAHIM *et al.*, 1971).

Anthocyanidin synthesis in tissue cultures, as in intact seedlings, is very much influenced by light. REINERT *et al.*, (1964) reported that anthocyanin synthesis in callus of *H. gracilis* only occurred in blue light. On the other hand STICKLAND and SUNDERLAND (1972b) demonstrated that white, blue, green and red light promoted anthocyanin synthesis. Blue light had the greatest effect, followed by white, red and green in that order. The latter authors suggested that the differences between their results and those of REINERT *et al.*, (1964) may have been due to different experimental conditions. It is interesting that studies with seedlings and segments of other species have also given conflicting results regarding the influence of different wavelengths of light (see STICKLAND and SUNDERLAND 1972b). This may be understood when it is appreciated that light is only one of several factors which influence pigment accumulation.

In *H. gracilis* callus it was found that in blue light the ratio of cyanidin-3-glucoside to cyanidin-3-rutinoside was initially 5:1 but changed to 1:1 during the growth period (STICKLAND and SUNDERLAND 1972b). It was suggested that there was an initial rapid synthesis of the 3-glucoside accompanied by a slower conversion to the rutinoside. The cessation of the short-lived rapid pigment formation in blue light was thought to be due to the absence of a dark period which was essential for the replenishment of precursors or possibly the regeneration of the photo-receptor system. It was supposed that the demands on precursors or photosystem would be less in red light, where anthocyanin synthesis is slower. There is then, evidence that anthocyanin biosynthesis may be controlled by a high energy photoreaction and a low energy red/far red reversible phytochrome reaction. Similar conclusions have been drawn from experiments with intact seedlings and segments.

In studies with batch suspension cultures of *H. gracilis* it has been shown that the stage of development of the culture is important in determining the production of anthocyanins (FRITSCH *et al.*, 1971). The initiation of anthocyanin synthesis was earlier, and the production greater, in cultures which had been kept in

the dark three days prior to illumination, than in cultures which had been kept in the dark for only one day prior to illumination.

BLAKELY and STEWARD (1961) found that anthocyanidin production in callus of *H. gracilis* was markedly influenced by the medium constituents, particularly the auxin concentration. In a more detailed study CONSTABEL *et al.*, (1971) showed that high concentrations of NAA and 2,4-D suppressed anthocyanin accumulation in cell suspensions of the same species, but that the total amount of pigment produced was unaffected due to differences in growth rate. Similarly STICKLAND and SUNDERLAND (1972a) observed that 2,4-D added to the medium reduced the amounts of anthocyanin produced by callus of *H. gracilis*. In contrast ALFERMANN and REINHARD (1971) were able to replace the light requirement for anthocyanin biosynthesis in cultures from *D. carota* with auxin. Dark-grown unpigmented cells synthesised anthocyanins when transferred to media containing 2,4-D at 10^{-5}M, IAA at 10^{-4}M, or NAA at 10^{-4}M. However the patterns of accumulation for light-induced and auxin-induced anthocyanin synthesis were quite different. Anthocyanins were detectable 3 days after exposure to light, but after 6 days of auxin treatment. Furthermore, low concentrations of 2-thiouracil or cycloheximide inhibited 2,4-D dependent pigment formation, but stimulated synthesis in the light. It was therefore concluded that at least 2 mechanisms are involved. On the other hand the presence of gibberellic acid in the medium inhibited both light-induced and auxin-induced anthocyanin formation (SCHMITZ and SEITZ, 1972). Similarly GREGOR (1974) found that the presence of gibberellic acid at 10^{-6} g/ml during irradiation with blue light completely inhibited anthocyanin production in cultures of *H. gracilis*. Gibberellic acid also reduced the PAL activity by 60% in blue light, but only by 35% in the dark. Other factors which influence anthocyanin accumulation in cultures include sucrose concentration (BALL and ARDITTI, 1974) and nitrogen nutrition (HELLER, 1948).

Several authors have investigated the relationship between PAL activity, a key enzyme, and the production of anthocyanins. For example GREGOR and REINERT (1972) showed that exposure to blue light increased PAL activity and subsequent anthocyanin production in callus of *H. gracilis*. However the situation was complicated by the observation that the response changed during the growth period, and that PAL activity was significant even in dark-grown cultures. The largest effect of blue light was seen in the cultures three weeks after transfer; when irradiation for 48 h resulted in a 400% rise in PAL activity followed by a gradual decline. Actinomycin D and puromycin treatments inhibited the blue-light-induced activity, and there was no evidence of a PAL-inactivating system. Irradiation with red light alone, or given after induction with blue light, had no effect on PAL activity in this system. In a less detailed study CONSTABEL *et al.* (1971) were unable to show a correlation between PAL activity and anthocyanin accumulation in suspension cultures of *H. gracilis*. However, in all these studies it should be appreciated that PAL is not only a key enzyme for the biosynthesis of anthocyanins, but also for the biosynthesis of other phenylpropanoid compounds such as cinnamic acids, coumarins and lignins. It would therefore be surprising if PAL activity was only correlated with anthocyanin production.

Little work has so far been done on the details of anthocyanin biosynthesis, but FRITSCH *et al.* (1971) have shown that phenylalanine, dihydrokaempferol, and

4,2′,4′,6′-tetrahydroxychalcone are incorporated into cyanidin in suspension cultures of *H. gracilis* exposed to blue light.

It is clear from this discussion that different tissue cultures produce a wide variety of flavonoids, and may be used effectively for both physiological and biochemical studies. However, it should be noted that the flavonoids produced are not always those which are characteristic of the entire plants from which the cultures were derived.

4.3 Tannins and Tannin Precursors

Although tannins are common constituents of cultured tissues, there have been relatively few detailed studies of the processes involved in their biosynthesis and accumulation. CONSTABEL (1965) investigated the tannins in callus tissues of *Juniperus communis* and found that they constituted about 13% of the total dry weight, and included both hydrolyzable and condensed tannins. The tannins were found either in isolated cells scattered throughout the callus or in groups of cells. The content of tannins increased during the first 30 days in culture, when the growth rate was low, but then decreased as the rate of cell division increased. The accumulation of tannins was enhanced by adding likely precursors, such as cinnamic acid, ferulic acid and sinapic acid to the culture medium, but since these acids also reduced the growth rate it was not certain that their effects were directly on tannin biosynthesis. Tissues cultured in the dark also showed increased tannin production, but again this could have resulted from the reduced growth rates. CONSTABEL (1965) came to the general conclusion that tannin content was inversely related to growth rate.

In studies of the polyphenol metabolism in callus cultures derived from *Camellia sinensis*, FORREST (1969) has identified catechin, epicatechin and leucoanthocyanins (potential precursors of condensed tannins) together with oligomeric and polymeric leucoanthocyanins. However, he found that the callus cultures did not synthesise the complex catechins which are characteristic of the intact tissues. Although exposure to light caused a several-fold increase in all the monomeric compounds, it appeared to inhibit polymerisation of the leucoanthocyanins. The activity of polyphenol oxidase, a probable initiator of polymerisation, was inversely correlated with the growth rate. It was concluded that, in general, decreased growth rates caused by various environmental conditions, such as intense light or nutritional abnormalities, gave rise to increased polyphenol synthesis.

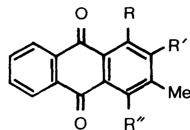
DAVIES (1972) studied polyphenol biosynthesis in cell suspension cultures of Paul's Scarlet Rose, and found that polyphenol accumulation was essentially restricted to late and post exponential growth phases. Of 14 polyphenols detected D-glucogallin, gallotannins, epicatechin and catechin were tentatively identified. After a series of experiments it was concluded that the initial rate of synthesis was largely influenced by a complex of factors including auxin concentration in the medium and light intensity, while the duration of synthesis was largely determined by the availability of carbohydrates. High light intensities partially reversed the inhibition of polyphenol synthesis caused by 5×10^{-5}M, 2,4-D and enhanced accumulation at 5×10^{-7}M, 2,4-D. In a later paper DAVIES (1972a)

found a close correlation between PAL activity and polyphenol synthesis under a wide variety of cultural conditions, although there was some discrepancy in timing between maximum rate of polyphenol accumulation and the period of maximum enzyme activities.

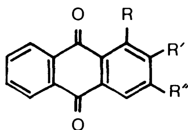
Fine structural aspects of tannin production have been examined in callus derived from *Pinus elliotti* (BAUR and WALKINSHAW, 1974). Tannins were found in association with smooth endoplasmic reticulum, in vesicles, and within cell vacuoles. While tannin accumulation was often accompanied by a generalised degradation of the cytoplasm, this was not always the case.

5. Anthraquinones and Naphthoquinones

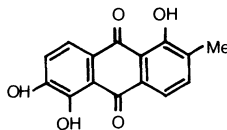
Although small in number, the reports on the presence of anthraquinones in plant tissue cultures have been encouraging. For example digitolutein, 3-methylquinizarin, pachybasin, 3-methylalizarin and 2 new compounds, 4-hydroxydigitolutein and 3-methylpurpurin (Fig. 5A), have been isolated from callus cultures of *Digitalis lanata* (FURUYA and KOJIMA, 1971; FURUYA *et al.*, 1972). It was interesting that 3-methylpurpurin and 3-methylalizarin were not detected in the shoots of intact plants. In another study, morindone, alizarin and damnacanthal (Figs. 5B and C) were isolated from suspension cultures of *Morinda citrifolia* (LEISTNER, 1973). Again the patterns of compounds produced differed from those found in the intact plants. While damnacanthol was the major compound in the cultures,



A.		R	R′	R″
i	Digitolutein	MeO	OH	H
ii	4 - Hydroxydigitolutein	MeO	OH	OH
iii	3 - Methylquinizarin	OH	H	OH
iv	Pachybasin	OH	H	H
v	3 - Methylalizarin	OH	OH	H
vi	3 - Methylpurpurin	OH	OH	OH



B		R	R′	R″
i	Alizarin	OH	OH	H
ii	Damnacanthal	MeO	CHO	OH



C. Morindone

Fig. 5A–C. Anthraquinones found in callus tissues of *Digitalis lanata* (A) and *Marinda citrifolia* (B and C)

morindone was dominant in one-year-old plants. In addition, alizarin was absent in the latter. Experiments with ^{14}C labelled precursors, such as mevalonic acid, acetate, shikimic acid and O-succinoylbenzoic acid, indicated that morindone and alizarin were derived from shikimic, glutamic and mevalonic acids by way of O-succinoylbenzoic acid and unknown naphthalene intermediates, and not via the malonate pathway. This is in contrast to the biosynthesis of anthraquinones found in fungi and lichens, which are thought to be formed by the cyclization of extended polyketides derived from the acetate-malonate pathway.

The anthraquinones, chrysophanol, physcion, rheum emodin, aloe emodin and rhein have been detected in callus cultures of *Cassia angustifolia* and *Cassia tora* (FRIEDRICH and BAIER, 1973; TABATA *et al.*, 1975). In the case of *C. tora* the content of total anthraquinones in the callus was higher than in dry seeds. Furthermore it was shown that the concentrations of auxins and cytokinins in the medium influenced the production of total anthraquinones. These effects, however, were quantitative rather than qualitative.

The presence of plumbagin (5-hydroxy-2-methyl 1,4-naphthoquinone) has recently been detected in callus cultures of *Plumbago zeylanica* (HEBLE *et al.*, 1974). The plumbagin content was highest in an anthocyanin pigmented strain where there was 135 mg/100 g fresh weight compared with 650 mg/100 g fresh weight in intact roots. Apparently the induction of roots in another "nodular" strain did not influence the amounts of plumbagin produced.

6. Isoprenoids

6.1 Latex

While there have been several attempts to use tissue cultures for studying the formation of latex, the results so far have been disappointing (KRIKORIAN and STEWARD, 1969). Of the many callus cultures derived from plants which are known to produce latex, e.g. *Euphorbia marginata*, *Parthenium argentatum*, there are few, if any, reports of the production of significant amounts of latex in established cultures. As pointed out by KRIKORIAN and STEWARD, laticiferous vessels and ducts are so complex that it is to be anticipated that these systems will present special difficulties under cultural conditions.

6.2 Monoterpenes

The older studies indicated that calli derived from *Mentha piperita*, *Mentha spicata* and other species do not produce detectable quantities of the monoterpenes characteristic of the whole plant (see KRIKORIAN and STEWARD, 1969). More recently BECKER (1970) has surveyed cultures from *Pimpinella anisum*, *Foeniculum vulgare*, *Levisticum officinalis*, *M. piperita*, *M. pulegium*, *Origanum vulgare*, *Salvia officinalis* and *Rosmarinus officinalis* for their volatile oil contents. None of the calli which lacked organised structures gave steam distillates with component

spectra comparable to those of the intact plants. Similarly it has been shown that callus cultures of *Nepeta cataria* do not synthesise the methylcyclopentane monoterpenes, e.g. nepetalactone, found in the intact plants (DOWNING and MITCHELL, 1975).

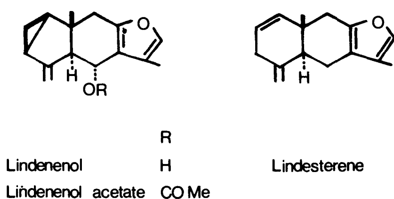
It has also been shown that cultures derived from a number of rose varieties do not produce monoterpenes (MACRAE and HOW, via JONES, 1974). This perhaps was not surprising since the cultures did not possess the specialised epidermal cells which are associated with the production of monoterpenes in intact petals. Nevertheless these workers did some very interesting experiments with precursors on the cultures from the Lady Seton Rose. It was discovered that the addition of geraniol, but not geranyl pyrophosphate, to the medium induced the cultures to produce geranial neral, nerol and citronellol. The aldehydes appeared very rapidly to reach their peaks within an hour. On the other hand the amounts of nerol and citronellol increased more slowly, reaching maximum concentrations after 6 h and then declining to zero after about 24 h. It was notable that all these compounds were present in the medium at higher levels than in the cells. In addition, β-glucosides of the monoterpene alcohols were found both in the cells and in the medium. Since the pattern of synthesis observed was similar to that described for intact petal tissues, it was tentatively suggested that the absence of monoterpenes in the cultures resulted from the lack of geranyl pyrophosphatase activity. However, it has been pointed out that even if the enzyme was present, it would not guarantee that the rates of synthesis would exceed rates of degradation.

It is clear from all these studies that callus cultures do not usually synthesise significant quantities of monoterpenes, which are normally produced within or in association with specialised cells.

6.3 Sesquiterpenes

Like the monoterpenes, the volatile sesquiterpenes are often components of essential oils, and like the former they are not often produced by callus cultures. For example, callus cultures of *Pogostemon cablin* did not contain detectable quantities of the sesquiterpenes typical of the parent plant (HART *et al.*, 1970). Similarly, callus tissues of *Daucus carota* did not form daucol or carotol (CONNOLLY and BUTCHER unpublished). Nevertheless there have been some reports of the presence of sesquiterpenes in cultures. Callus of *Lindera strychnifolia*, which had been established for a year, were found to produce lindenenol, lindenenol acetate, linderane, linderalacton, lindesterene, and caryophyllene (Fig. 6) (TOMITA *et al.*, 1969). The proportions of the different compounds differed markedly from those of the original plants. The callus cultures produced nearly equal amounts of lindenenol, lindenenol acetate, linderane and linderalacton, while lindenenol acetate was the dominant sesquiterpene in intact leaves and shoots. Here it should be noted that these oxidized sesquiterpenes are probably distributed throughout the plant, and not synthesised or accumulated in specialised cells.

Relatively large amounts of sesquiterpenes have also been found in callus and suspension cultures derived from *Andrographis paniculata*, a plant which is known to produce the diterpene andrographolide and related compounds (ALLISON *et*



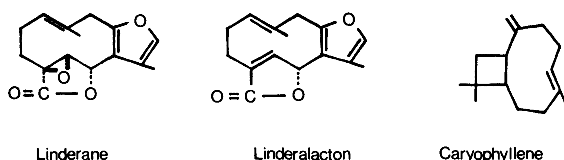


Fig. 6. Sesquiterpenes found in callus cultures of *Lindera strychnifolia*

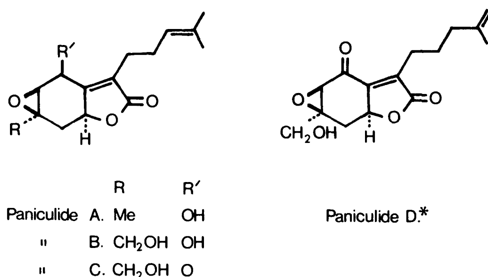


Fig. 7. Sesquiterpenes produced by suspension cultures of *Andrographis paniculata*. (*CONNOLLY unpublished)

al., 1968; BUTCHER and CONNOLLY, 1971). The sesquiterpenes which had not previously been described were called paniculides A, B, C, and D (Fig. 7). The paniculides, although present in the tissues, were mainly secreted into the medium. Cultures derived from leaves, stems, hypocotyls, roots and embryos all produced paniculides, but no andrographolides were detected. Conversely andrographolides, but no paniculides, were found in the leaves, stems and roots of intact seedlings. The pattern of the sesquiterpenes produced in the cultures did not appear to be influenced by medium constituents, but exposure to light altered the proportions of the compounds present.

Recently it has been shown that cell-free extracts from suspension cells of *Andrographis paniculata* incubated with [2-^{14}C] mevalonate produce labelled *trans*, *trans* and *cis*, *trans* farnesols and γ-bisabolene. This system is currently being used to investigate the possible interconversions of the two farnesol isomers via the aldehydes (OVERTON and ROBERTS, 1974a and b).

6.4 Diterpenes

There are very few reports of the presence of diterpenes in callus and suspension cultures. As mentioned in the previous section callus and suspension cultures of *A. paniculata* did not produce the diterpene andrographolide or related com-

pounds which are characteristic of the intact plant (BUTCHER and CONNOLLY, 1971). In this case the diterpenes are normally distributed throughout the plant, and their synthesis and accumulation do not appear to be associated with specialised cells. This may be an example where the organisation of the tissues is important for the production of the characteristic secondary product. Further unpublished investigations by these authors have indicated that several other cultures do not produce detectable quantities of the diterpenes characteristic of the entire plants. They found that callus cultures of *Delphinium ajacis*, *Swietenia mahogani* and *Taxus baccata* did not accumulate ajaconine, swietenine and taxicin respectively.

6.5 Sterols and Triterpenes

The phytosterols are ubiquitous in higher plants and probably also in plant tissue cultures. Indeed it is probable that they are essential components of many cellular membranes. Thus in the strict sense they may not be secondary products as defined at the beginning of this article. However, their role has not been unequivocally established, and the kinds and prôportions of the sterols vary considerably from one plant species to another. The steroids found in callus and suspension cultures have recently been reviewed (STOHS and ROSENBERG, 1975). As in intact plants, the most widespread sterols are β-sitosterol, stigmasterol and campesterol. These have been isolated from cultures of *Nicotiana tabacum* (BENVENISTE *et al.*, 1966), *Dioscorea tokoro* (TOMITA *et al.*, 1970), *Withania somnifera* (YU *et al.*, 1974), Paul's Scarlet Rose (WILLIAMS and GOODWIN, 1965), *Tylophora indica* (BENJAMIN and MULCHANDANI, 1973) and *Helianthus annuus* (BUTCHER *et al.*, 1974). Other sterols and tetracyclic, triterpenes detected include cycloartenol, 2,4-methylene cycloartenol, citrostradienol,citrostradiol, cycloeucalenol and obtusifoliol in *N. tabacum* cultures (BENVENISTE *et al.*, 1966; BENVENISTE, 1968), 2,4-ethylidene cholesterol and 2,4-methylene cholesterol in callus of *W. somnifera* (YU *et al.*, 1974), isofucosterol and cholesterol in callus of *Helianthus annuus* (BUTCHER *et al.*, 1974). In addition, the pentacyclic triterpene, β-amyrin, has been found in Paul's Scarlet Rose and *T. indica* (WILLIAMS and GOODWIN, 1965; BENJAMIN and MULCHANDANI, 1973) and arundoin has been detected in callus of *Oryza sativa* (YANAGAWA *et al.*, 1972).

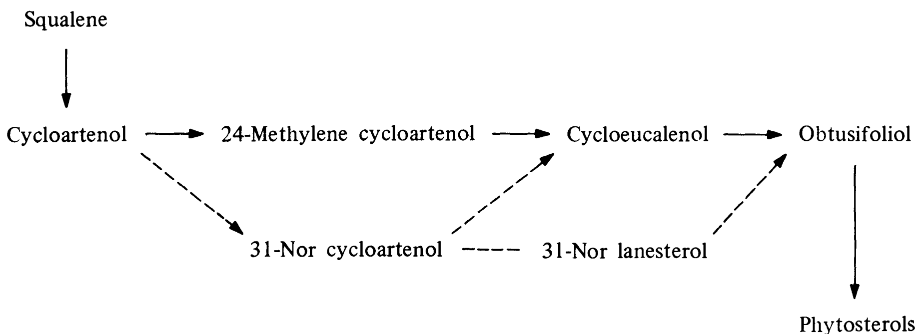


Fig. 8. Scheme proposed for the biosynthesis of phytosterols in *Rubus fruticosus*

Cultures of *N. tabacum* and *Rubus fruticosus* have provided excellent experimental systems for studying phytosterol biosynthesis. When ^{14}C labelled acetate, mevalonate and other sterol precursors are incubated with callus tissues they are efficiently incorporated into the intermediates of the sterol biosynthesis (BENVENISTE, 1968; BENVENISTE *et al.*, 1966; HEINTZ *et al.*, 1972a, b). After a detailed series of studies the scheme shown in Figure 8 was proposed as the pathway, for sterol biosynthesis. This pathway if correct, would explain the occurrence of cycloeucalenol and obtusifoliol and the absence of lanesterol in many higher plants.

6.6 Steroidal Alkaloids

Tomatine, a glycoside of the steroidal alkaloid tomatidine, has been isolated from excised roots of *Lycopersicum esculentum* (SANDER, 1956; RODDICK and BUTCHER, 1972a, b; RODDICK, 1974). The tomatine content, however, in excised roots was much lower than in seedling radicles of the same age. Tomatine has also been isolated from newly initiated callus cultures of tomato, but here it was not possible to exclude the possibility that the alkaloid originated from root primordia which were present in these cultures (RODDICK and BUTCHER, 1972a). Only one of several long-established callus isolates, which did not form roots, produced tomatine, and even then it was only in very small amounts.

Solasonine, a glycoside of solasodine, has been detected in callus cultures of *Solanum xanthocarpum* (HEBLE *et al.*, 1971). In this case the type of plant hormone (IAA, indolebutyric acid or gibberellic acid) in the medium, but not the formation of roots, appeared to affect the secondary metabolites produced. In another study solasodine was not found in callus cultures of *Solanum laciniatum*, although it was a characteristic component of the entire plant (VÁGÚJFALVI *et al.*, 1971).

6.7 Carotenoids

The carotenoid pigments have a wide distribution in higher plants and are known to be components of chloroplasts and other plastids where they may have important functions. Thus, as might be expected, callus cultures which have chloroplasts also contain carotenoids. In these cases the ratios of the various pigments including chlorophylls and carotenoids are often similar to those found in the young leaf primordia in which the chloroplasts are immature (SUNDERLAND, 1966). Unlike the storage roots from which they are derived, carrot root callus cultures usually do not have chromoplasts with high levels of carotenoids. However, there have been reports of the isolation of orange carrot strains which produce relatively large amounts of carotene and xanthophyll (NAEF and TURIAN, 1963).

The carotenoids in stem callus of Paul's Scarlet Rose have been identified as zeaxanthin, violaxanthin, auroxanthin and neoxanthin (WILLIAMS and GOODWIN, 1965). The total concentration in the cultures was four times less than in the stem, and 30 times less than in leaves. The pattern of carotenoids produced by the callus

was also quite different from that of the intact tissues. Similarly the levels of carotenoids in callus from *Ruta graveolens* were considerably less than in intact leaves, but here the proportions were approximately the same (SCHARLEMANN and CZYGAN, 1971). The compounds identified were β-carotene, lutein, zeaxanthin, lutein-5,6 epoxide, antheraxanthin, violaxanthin and neoxanthin.

7. Unusual Fatty Acids and Related Compounds

This area of secondary metabolism has not been studied extensively, but there have been a few significant investigations. Sterculic acid, dihydrosterculic acid, malvalic acid and dihydromalvalic acid (Fig. 9) have been isolated from callus cultures derived from *Malva paviflora* and *Malva sylvestris* (YANO *et al.*, 1972a). With the use of radioactive precursors it was possible to establish the course of the biosynthesis of these compounds in the cultures and the scheme shown in Figure 10 was proposed (YANO *et al.*, 1972b).

Small amounts of the cyclopentenyl fatty acids, hydnocarpic, chaulmoogric and gorlic acids have been found in callus cultures of *Hydnocarpus anthelminthica* (SPENER *et al.*, 1974). However in this instance callus cultures were considered to be unsuitable for studying the biosynthesis of these acids, which normally accumulate in large amounts in the seeds of this species.

Another unusual fatty acid, erucic acid [$CH_3(CH_2)_7CH=CH(CH_2)_{11}COOH$], which is often found in crucifer seeds, was not detected in disorganised callus tissues from turnip rape *Brassica rapa* (STABA *et al.*, 1971). It is interesting to note

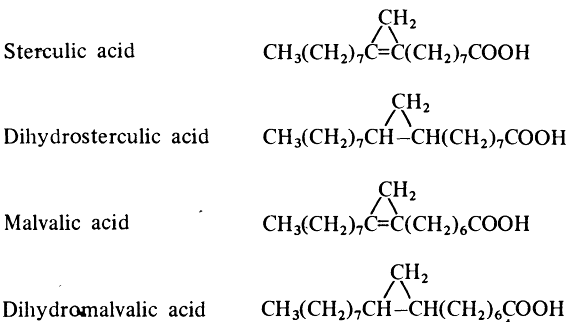


Fig. 9. Cyclopropane and cyclopropene fatty acids found in callus cultures of *Malva paviflora* and *Malva sylvestris*

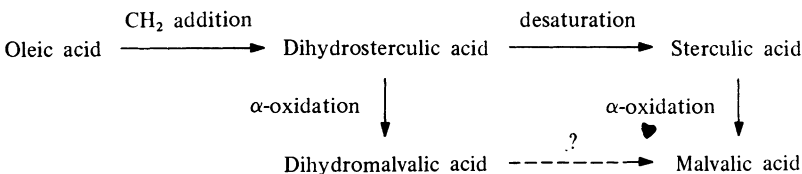


Fig. 10. Scheme proposed for the biosynthesis of cyclopropane and cyclopropene fatty acids in *Malva paviflora* and *M. sylvestris*

that callus cultures of *Crambe abyssinica* (Cruciferae) which formed embryolike structures did produce small amounts of erucic acid (JONES, 1974).

Long chain fatty acids (up to 26 carbons) have been found in callus cultures of *Daucus carota* and *Hydrocarpis anthelminthica* and suspension cultures of *Phaseolus aureus*, *Cicer arietinum* and *Petroselinum hortense* (RADWAN *et al.*, 1974). It was suggested that cultural conditions inhibit fatty acid desaturation and promote elongation, since the amounts of these long chain fatty acids were highest in lipids containing the lowest levels of unsaturated acids. These long chain fatty acids were found predominantly as constituents of steryl esters and esterified steryl glycosides.

In an investigation of the aliphatic hydrocarbons of tobacco cultures it was discovered that the patterns of compounds produced differed markedly in crown-gall teratoma and habituated calli (WEETE *et al.*, 1971). The teratoma cultures, which possessed a certain amount of tissue organisation, contained n-C_{29}, 2-methyl C_{30}(iso C_{31}) alkanes, which were qualitatively similar to those found in intact seedlings. On the other hand, habituated cultures, which were completely disorganised, contained a different population of alkanes ranging from C_{17} to C_{28}. This example again seems to point to the importance of the presence of organised structures for the production of certain secondary products.

Another interesting investigation has shown that callus cultures of *Ruta graveolens* produce relatively large amounts of volatile oils (REINHARD *et al.*, 1971; CORDUAN and REINHARD, 1972). In light-grown calli the major components were 2-undecanone and 2-undecanyl acetate with smaller amounts of 2-nonanone, 2-nonanyl acetate, 2-nonanol and 2-undecanol. These compounds were excreted into schizogenous passages. The anatomical features of the secretory passages in the callus resembled those in leaves, green stems and fruits. The oil, to some extent, was also excreted into the parenchyma cells in the form of small droplets. The composition of the oil in light-grown callus most closely resembled the oil found in leaves. On the other hand, the oil of dark-grown cultures resembled that found in the roots and stems covered by bark where the oil is distributed in apparently unspecialised cells. It is interesting to note that different nutrient media did not influence the composition of the volatile oil and that the light effect did not depend on photosynthesis. These studies were very significant since they represent one of the few reports that volatile oils are produced in culture. However, it should be appreciated that specialised cells capable of accumulating large amounts of oils, comparable to those in intact plants, are present in these cultures. Hence these findings are not against the general view that specialised cells are required for the production of large amounts of volatile oils in tissue cultures.

8. Miscellaneous Products Derived from Amino Acids

8.1 Glucosinolates (Mustard Oil Glycosides)

These compounds are characteristic of the family Cruciferae and a few related families. The glucosinolates form a large group of compounds which are synthesised from a wide range of common and rare amino acids.

Examinations of callus from *Iberis sempervirens* have failed to reveal the presence of glucoibervirin, glucoerucin and glucoiberin, which are found in the entire plants (KRIKORIAN and STEWARD, 1969). Similarly none of the characteristic glucosinolates were found in suspension cultures of *Armoracia lapthifolia, Eruca sativa, Iberis sempervirens, Nasturtium officinale* and *Sinapis alba* (KIRKLAND *et al.*, 1971). However, myrosinase, an enzyme capable of hydrolysing the glucosinolates, was detected. On the other hand, the same authors did find benzylglucosinolate in cell suspension cultures of *Tropeaolum majus* and 2-phenylethylglucosinolate and 2-hydroxy-2-phenylethylglucosinolate in suspension cultures of *Reseda luteola*, but the relative amounts of the glucosinolates in the cultures were quite different from those in the intact plant.

In contrast, clonal excised roots of *Isatis tinctoria* produced the indole glucosinolates, glucobrassicin and neoglucobrassicin in amounts similar to those found in intact roots (ELLIOTT and STOWE, 1971). The same compounds have been isolated from clonal excised roots of *Brassica rapa* (EL-TIGANI, 1972). The indole glucosinolates were also detected in newly initiated callus cultures of *B. rapa*, but since root primordia were also present, it was not certain whether or not glucosinolate production depended on the presence of organised structures. Four-year-old callus isolates with no organised structures failed to produce glucosinolates. Here it seems that glucosinolates, characteristic of the intact plants, are often synthesised by excised roots, which retain the normal organisation of intact roots, but their production in callus cultures is less certain.

8.2 Alliins

The alliins are characteristically found in the Liliaceae and are precursors of the substances which are responsible for the odour, flavour and lachrymatory activity of onions.

Recently it was shown that ten-month-old callus cultures derived from the basal disc of *Allium cepa* only produced significant amounts of the characteristic disulphides, resulting from alliin degradation by alliinase, when roots were present (FREEMAN *et al.*, 1974). Experiments with alliinase and the synthetic alliin, ($\pm$)S-n-propyl-L-cysteine sulphoxide, suggested that the lack of flavour components in calli lacking roots resulted from the absence of precursors (alliins) rather than alliinase. This, then, appears to be another example where the production of particular secondary products depends on the presence of organised tissues.

9. Conclusions and Prospects

As anticipated in the earlier part of this article, the successful use of plant tissue cultures in biosynthetic studies depends very much on the factors which influence the production of the desired compounds in the intact plant. Of these factors, the most important is probably the closeness of the relationship between the production of the compounds and differentiation. Indeed in many cases secondary product biosynthesis may be considered to be part of the differentiation process.

From the point of view of using tissue cultures for biosynthetic studies, secondary products may be conveniently divided into 4 broad categories. Firstly there are the compounds such as the simple coumarins, certain flavonoids, and phytosterols, which have a widespread occurrence in the plant kingdom, and which do not seem to be associated with any specialised differentiated structures. These are frequently found in tissue cultures, although the compounds produced may be both qualitatively and quantitatively different from those found in the entire plant. This is well illustrated with the anthocyanins, where the compounds produced by the cultures may be different or even absent in the intact plant.

Secondly there are the widely distributed compounds which are normally associated with cell types which are frequently found in cultures e.g. lignins and tannins. These compounds are often encountered in tissue cultures, particularly when the appropriate cell types are present. As in the first category there are often differences between the compounds found in the plant and those in tissue cultures. A good example of this is seen with lignins extracted from calli derived from angiosperms in which the extracted material resembles conifer lignin. Furthermore the lignins in tissue cultures are not always confined to the cell walls, and are often located in intercellular spaces.

A third very broad category includes compounds which have a restricted distribution in the plant kingdom, but whose synthesis and accumulation do not appear to be associated with any specialised cell types. Within this category the production of the required compounds is unpredictable. On the positive side we have cultures of *Ruta graveolens*, *Petroselinum hortense* and *Digitalis lanata*, which produce furanocoumarins, specific flavonoids and anthraquinones respectively, classes of compounds which are characteristic of the entire plants. Again there are usually differences in the patterns of compounds produced, and indeed compounds not detected in the plant have been isolated. On the negative side we have callus cultures of *Allium cepa*, *Andrographis paniculata* and *Brassica rapa* which do not produce significant quantities of the alliins, diterpenes and indole glucosinolates respectively. In between these extremes there are cultures which produce the compounds characteristic of the entire plant, but in very low yield. In some ways this category is the most interesting, since it includes many compounds of commercial and medicinal significance. Unfortunately it is also the most unpredictable group.

The fourth category includes those compounds whose synthesis and accumulation is normally associated with specialised cells or groups of cells. It includes constituents of most essential oils, resins and latex. These compounds are only rarely found in callus and suspension cultures, presumably because their synthesis and accumulation is completely dependent on highly specialised cells which are not found in the cultures. An exception to this generalisation is seen with the production of significant quantities of volatile oils by callus of *R. graveolens*. Success in this case is likely to be due to the fact that cells resembling the oil cells in the intact plant occur in these calli. This suggests that other compounds in this category may be produced in culture if the appropriate structures can be induced.

While discussing the significance of differentiation and organised structures to secondary product biosynthesis, it is appropriate to recall that organ cultures such as excised roots, which retain the fundamental organisation of the intact organ also frequently produce the compounds characteristic of the plant. For

example, excised roots of *Isatis tinctoria* and *B. rapa* produce indole glucosinolates, while excised roots of *Lycopersicum esculentum* produce steroidal alkaloids.

The reason why differentiation is important for secondary product biosynthesis is at present a matter of speculation. However, it seems likely that features such as the spatial arrangement of enzymes, the presence of specific organelles, compartmentalisation of enzymes and substrates, and the locations available for deposition, determine whether or not a specific compound is produced. For instance compartmentalisation may be important for separating degradative enzymes from the accumulated metabolites, since it is well known that intact tissues often possess enzymes capable of degrading the metabolites they accumulate. For example, *Allium* sp. possess the enzyme alliinase which degrades the alliins, and crucifers possess myrosinase which degrades glucosinolates. If there is no separation of these substrates and enzymes in cultural conditions, these secondary metabolites would not be expected to accumulate. Similarly, a suitable location for deposition, e.g. cell walls, cell vacuoles, resin ducts, and latex vessels, may be essential for the accumulation of products, particularly when such compounds are toxic to the cytoplasm. If cultured tissues do not form the structures where toxic materials are normally deposited, only those cells which produce non-toxic levels of the compounds will survive. If this is correct, the cultural procedures may select cells which lack the ability to accumulate significant amounts of the desired products. In some cases this problem may be overcome if the liquid medium can serve as an alternative place for deposition of toxic compounds. It is perhaps significant that the paniculides, which are toxic compounds, are excreted into the medium of suspension cultures of *Andrographis paniculata*. There are several other examples in which secondary products are excreted into the medium.

In contrast to the possibilities discussed above, secondary product synthesis may sometimes be a part of the differentiation program of a particular cell type, and not dependent on the resulting structure of the cell. If this is the case, then it may be possible to select mutants in which the "required" metabolic pathway is separated from the differentiation program with which it is normally associated. The highly pigmented strains of *Haplopappus gracilis* and *Daucus carota* may represent mutants of this type.

With some secondary metabolites the actual organisation of the tissues may be important, particularly where precursors and products are synthesised in different tissues of the same organ or in different organs. Here it could be envisaged that a particular arrangement of tissues or organs would facilitate the transport of precursors and therefore influence the synthesis of the final product. This may be the situation in cases where the desired product is not formed in callus in spite of the fact that synthesis in intact tissues is not associated with specialised structures.

Differentiation and tissue organisation are not the only factors which influence the synthesis of secondary metabolites. Environmental factors, particularly light, can have a marked influence on the production of certain secondary metabolites. This is well illustrated with the biosynthesis of flavones and flavonols in suspension cultures of *Petroselinum hortense*, anthocyanins in callus of *H. gracilis* and volatile oils in callus of *R. graveolens*. Further, medium constituents, particularly plant hormones, often have a dramatic effect on the type and pattern of products produced. However, it is not always easy to decide whether these

effects are direct or result from the effects of these compounds on growth and differentiation. As could be anticipated, the addition of precursors to the medium may also enhance the amounts of secondary products formed.

Clearly in the last few years there has been notable success in the use of tissue cultures for biosynthetic studies of secondary metabolites. In particular, the studies of the biosynthesis of furanocoumarins, anthraquinones, cyclopropane fatty acids and phytosterols have provided adequate evidence of the effectiveness of tissue culture techniques. The physiological studies of the production of anthocyanins and lignins have also yielded valuable information. It may be anticipated that in the next few years the continuous culture techniques will begin to be exploited for studies of the factors which determine secondary product metabolism. With such culture systems it should be possible to obtain fundamental knowledge about the relationships between primary and secondary metabolism, an area of research which has so far been neglected.

The discovery that certain tissue cultures produce compounds which have not previously been described, e.g. paniculides, rutacultin, was unexpected, but suggests that cultures may provide an important source of new compounds.

So far there have been no reports that tissue cultures are being used for the commercial production of compounds. This perhaps is not surprising, since the requirements for this are very stringent. Not only do the cultures have to synthesise the desired compounds, but they also have to produce them more cheaply than the usual commercial sources. This pre-requisite is likely to rule out the possibility of using tissue cultures for commercial biosynthesis of products except for the more expensive medicinal compounds. With the latter, success will depend very much on whether mutant strains producing high yields of the desired compounds can be isolated. A more promising possibility is that tissues may be useful for specific metabolic biotransformations which are difficult by other methods

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3. Tissue Culture and Pharmacy

E. J. STABA

1. Introduction

The advances in our understanding of useful product production from plant tissue culture systems have been numerous and encouraging. For example, it is now known that rapidly growing suspension cultures of *Ruta graveolens* simultaneously produce volatile oils (CORDUAN and REINHARD, 1972), furanocoumarins (AUSTIN and BROWN, 1973), and alkaloids (BOULANGER *et al.*, 1973). In Japan, *Phytolacca americana* suspension medium is presently being field tested for commercial use as a plant virus inhibitor (LIU, 1974). Perhaps also significant is the fact that patents have been applied for or granted to produce from plant tissue cultures diversified plant metabolites (METZ and LANG, 1966; ROBERT and McCORMICK, 1973; ROUTIEN and NICKELL, 1956), allergens (STABA and SHAFIEE, 1974), diosgenin (STABA and KAUL, 1971), L-dopa (BRAIN, 1974), ginsenosides (FURUYA and ISHII, 1972, 1973), and glycyrrhizin (TAMAKI *et al.*, 1973). These chemicals are used in medicine and pharmacy as therapeutic or diagnostic agents, as drug precursors, or for formulating and flavoring drugs.

Relatively recent reviews on this subject have been written by CAREW and STABA (1965), BECKER (1969), KRIKORIAN and STEWARD (1969), STABA (1969), PUHAN and MARTIN (1971), DOUGALL (1972), MANDELS (1972), STREET (1973c), and CONSTABEL *et al.* (1974). The author of this article was selective in the literature cited, although an effort was made to be comprehensive for tissue culture studies of the tropane alkaloids, ginsenosides, diosgenin, cardenolides, and biotransformation processes. For other details also see Chapter VI. 2 of this Volume.

2. Perspective

It is true that the need of greater knowledge and technology suggested more than a decade ago by NICKELL (1962) for profitable plant tissue culture product production has not yet been completely satisfied. However, it is obvious that the present cost restraint associated with plant tissue culture fermentation would be significantly reduced if a desired product was produced in an adequate amount in a reasonable time and at a low operational cost. Some of these requirements for commercial application have to some extent been satisfied.

The desirable products for production by plant tissue cultures are as diversified as is industry itself. Studies have been directed toward producing catharan-

thus (PATTERSON and CAREW, 1969) and camptotheca (ROBERT and MCCORMICK, 1973; MISAWA *et al.*, 1974) anti-tumor alkaloids; amino acids (LIU, 1974) and proteins (MANDELS *et al.*, 1968; PUHAN and MARTIN, 1971); antibiotics (KHANNA and STABA, 1968; VELIKY and GENEST, 1972), allergens (SHAFIEE and STABA, 1973; WU *et al.*, 1976), cigarette tobacco (ANONYMOUS, 1971), and fatty acids (SPENER *et al.*, 1974); papain (MEDORA *et al.*, 1973) and other enzymes (MANDELS *et al.*,1967; VELIKY *et al.*, 1969); steroid precursors (KAUL *et al.*, 1969) and steroid biotransformation products (FURUYA *et al.*, 1973; STOHS and EL-OLEMY, 1972a). Serious thought and attention have also been given to the production from plant tissue cultures of coloring agents, condiments and perfumes, food emulsifiers and sweeteners, insecticides, insulin-like compounds, and vitamins.

2.1 Amount of Products Produced

The amount of compounds such as diosgenin (KHANNA and MOHAN, 1973), ginseng sapoinins (FURUYA and ISHII, 1972; FURUYA *et al.*, 1973; JHANG *et al.*, 1974), glycyrrhizin (TAMAKI *et al.*, 1973), harmin (REINHARD *et al.*, 1968b), nicotine (SHIIO and OHTA, 1973), and rotenone (SHARMA and KHANNA, 1974) from tissue cultures has approached or exceeded that in the plant. Often, however, plant tissue cultures produce extremely low concentrations of the compound of interest (KRIKORIAN and STEWARD, 1969; STABA, 1969). It is nevertheless significant that serially propagated plant tissue cultures produce compounds such as anthraquinones (DHURA *et al.*, 1972; FURUYA *et al.*, 1972; LEISTNER, 1973; RAI and TURNER, 1974) and dianthrones (FRIEDRICH and BAIER, 1973); furanochromes such as visnagin (CHEN *et al.*, 1969) and furanocoumarins such as isopimpinellin (KUTNEY *et al.*, 1973) and psoralen (AUSTIN and BROWN, 1973); l-maackiain (FURUYA and IKUTA, 1968); pigments such as flavonoids (BRUNET and IBRAHIM, 1973; SAYAGAVER *et al.*, 1969) and anthocyanins (MATSUMOTO *et al.*, 1973); volatiles such as geraniol (SARDESAI and TIPNIS, 1969) and undecanone (CORDUAN and REINHARD, 1972); and the alkaloids reserpine (CAREW, 1965), berberine (FURUYA *et al.*, 1972) and caffeine (HALL *et al.*, 1974; KELLER *et al.*, 1972). Plant tissue cultures may very well contain metabolic pathways that have been modified and/ or abbreviated from that of the plant (CHEN *et al.*, 1969; MIZUSAKI *et al.*, 1971; NETTLESHIP and SLAYTOR, 1974). Opium poppy tissue cultures have produced a new alkaloid, norsanguinarine (FURUYA *et al.*, 1972) and latex-bearing cells persist for a period of time in *Hevea* tissue cultures (PARANJOTHY, 1974; WILSON, 1974). Tissue cultures of *Phaseolus* produce harmin, an alkaloid not present in the plant (VELIKY, 1972); *Andrographis* tissue cultures produce four new paniculide sesquiterpene lactones (BUTCHER, 1971); pheum tissue cultures produce compounds not present in the plant (RAI and TURNER, 1974); and tobacco tissue cultures produce new putrescine derivatives (MIZUSAKI *et al.*, 1971).

All of the above achievements are both encouraging and gratifying when one realizes that a decade ago one could not be confident that any unusual metabolite would be detected in a serially propagated plant tissue culture.

2.2 Factors Affecting Products Produced

Genetic, morphologic, and biochemical factors do affect plant tissue cultures and their ability to produce a significant amount of a product. The expression of these factors is undoubtedly interrelated.

2.2.1 Genetic

The genetic factors present within a plant species may be expressed differently while in tissue culture, and thus affect both the concentration and identity of products produced. It is well-known that many different plant species established as tissue cultures produce diosgenin (KAUL, 1969; MEHTA and STABA, 1970; TOMITA *et al.*, 1970; HEBLE *et al.*, 1971; KHANNA and JAIN, 1973; KHANNA and MOHAN, 1973; STOHS and ROSENBERG, 1974), nicotine (KRIKORIAN and STEWARD, 1969; KATO *et al.*, 1972), and tropane alkaloids (CHAN and STABA, 1965; KONOSHIMA *et al.*, 1967, 1970; THOMAS and STREET, 1970; DHOOT and HENSHAW, 1974).

The plant tissue part selected for culture may be critical for product production. It has been reported that the root-biosynthesized tropane (WEST and MIKA, 1957) and reserpine (MITRA and KAUL, 1964) alkaloids are best produced by root-derived callus tissue cultures. However, the tropane alkaloids (CHAN and STABA, 1965; KONOSHIMA *et al.*, 1970) and the root-synthesized product nicotine (SHIIO and OHTA, 1973; TABATA *et al.*, 1971) are produced from other than root-derived tissue cultures. The alkaloidal patterns of opium tissue cultures derived from either seedlings, roots, or stalk capsules are similar (FURUYA *et al.*, 1972). One must be alert to the fact that products may be carried over with the primary explant and may not persist on subculture (NETIEN *et al.*, 1967), or that the products produced might chemically change on repeated subculture (BOULANGER *et al*, 1973). Root, leaf, and stem callus cultures of *Murraya koenigii* did not contain, gradually lost, or persistently produced carbazoles, respectively (MITRA *et al.*, 1971). It is, however, well established that compounds such as the catharanthus (BODER *et al.*, 1964) and tobacco (SHIIO and OHTA, 1973) alkaloids, diosgenin (MEHTA and STABA, 1970), panoxasides (JHANG *et al.*, 1974), and visnagin (CHEN *et al.*, 1969) have persisted in callus cultures for many years.

It is not yet known if selecting tissues from a high product producing plant would result in a high product tissue culture. Tissue cultures that were established from seeds of high tropane alkaloid producing plants did not produce more tropane alkaloids (CHAN and STABA, 1965). However, it is a fact that the cultural conditions or gene expressions needed for datura tissue cultures to produce significant amounts of tropane alkaloids are unknown. Gene transformation and selection techniques for biochemical mutants have been studied with varying degrees of success for amino acid analogs (WIDHOLM, 1974), diosgenin (MEHTA *et al.*, 1972; SHAMINA and KARANOVA, 1974), and reserpine (SHAMINA *et al.*, 1971). Even if mutant strains were established, chromosomal populations are more often than not unstable (BAYLISS, 1974; CAREW and STABA, 1965; DOUGALL, 1972; SHAMINA and FROLOVA, 1974; SHERIDAN, 1974). Once the freezing technique for plant tissue culture is perfected (CAREW and STABA, 1965; Chap. VII. 3 of this Vol.), selected high product producing strains might possibly be conserved.

2.2.2 Morphology

The morphologic and cellular stage will affect the concentration of some tissue culture produced products. Callus tissue cultures may produce plant organs such as leaves, buds and roots (organized tissue), but also more difficultly observable cellular changes such as meristematic centers and vascular and lacticifer elements (unorganized, differentiated tissues). Callus cultures may also be uniformly parenchymatous and yet contain ultrastructural changes in the vacuoles, chloroplasts, amyloplasts, and other organelles (unorganized, undifferentiated tissues). Organization is believed more likely to occur from small cells containing starch than from large, highly vacuolated cells.

It is interesting to try and interpret the relationship of tissue culture morphology and cellular development to tropane biosynthesis. The belladonna tropane alkaloids are produced in significant concentrations by the initial and lateral roots of serially propagated organ cultures (MITRA, 1972), and to some extent from suspension cultures that form roots (THOMAS and STREET, 1970). However, belladonna (THOMAS and STREET, 1970), datura (CHAN and STABA, 1965; KONOSHIMA *et al.*, 1970; STOHS, 1969), hyoscyamus (DHOOT and HENSHAW, 1974), and scopolia (KONOSHIMA *et al.*, 1967) tropane alkaloids are poorly produced by callus tissue cultures. It has been suggested that the tropane alkaloid content of callus tissues would increase with the initiation of root or meristematic centers (STABA, 1969), or by having the tissue form root initials and/or small vacuoles (SIMOLA, 1972a). However, the tropane alkaloid content of hyoscyamus suspension cells with roots did not significantly increase, but did markedly decrease when cell aggregates were friable (DHOOT and HENSHAW, 1974). Although datura primary root organ cultures produce alkaloids (CAREW and STABA, 1965) and datura callus cultures can become organized (ENGVILD, 1973), no one has yet reported the alkaloid content of organized datura callus.

It is not essential that a tissue culture be organized to produce a product, as for example the production of diosgenin (KELLER *et al.*, 1972; SARKISOVA, 1974), ginseng triterpenoids (FURUYA *et al.*, 1970; JHANG *et al.*, 1974), and visnagin (CHEN *et al.*, 1969). It would be very interesting to know more about the mechanisms of subcellular production and the purpose of such compounds in the plant cell. It has been established that the mitochondrial, microsomal, and soluble fraction of dioscorea tissue cultures are more involved in cholesterol metabolism than the cell wall and nuclear fractions (STOHS and EL-OLEMY, 1972b). Stachydrine alkaloid production (SETHI and CAREW, 1974), but not tropane alkaloid (STABA and JINDRA, 1968) production has been correlated with the presence of chlorophyllous cells. Other subcellular/product correlations made are the association of the invertase enzyme with the cell wall fraction of carrot tissue cultures (UEDA *et al.*, 1974), and that the vacuoles of young dividing cells contain tannin (SIMOLA, 1972a) and occasionally other pigments (TABATA and MIZUKAMI, 1974).

Growth regulators do affect the morphologic and cellular state of a tissue culture and often, but not always, the concentration of a product produced. The growth regulator concentrations used in tissue culture systems vary significantly. Most investigators use a 2,4-dichlorophenoxyacetic acid (2,4-D) concentration range of 0.1 to 5.0 parts mg/l, however as high as 25 mg/l has been suggested for

the optimal production of L-dopa (BRAIN, 1974). Similarly, although kinetin is often used in concentrations less than 5.0 mg/l, as high as 30.0 mg/l has been used to induce tissue organization. Kinetin will stimulate both bud formation and nicotine production (TABATA *et al.*, 1971), but will decrease tropane alkaloid synthesis (KONOSHIMA *et al.*, 1970). Naphthaleneacetic acid will often promote and maintain root organ cultures (MITRA, 1968). It is well established that very low concentrations of 2,4-D will induce tissue cultures to organize (STABA *et al.*, 1965). The growth regulator, 2,4-D, will either totally (KATO *et al.*, 1972) or partially suppress (SHIIO and OHTA, 1973; TABATA *et al.*, 1971) nicotine production from tissue cultures. Carboline alkaloid production is totally inhibited by 2,4-D (NETTLESHIP and SLAYTOR, 1974), but diosgenin production is stimulated by low concentrations of 2,4-D (KAUL *et al.*, 1969). Indoleacetic acid will increase diosgenin but suppress solasadine production in solanum tissue cultures (HEBLE *et al.*, 1971). Enzymes, such as peroxidase, may inactivate the auxin indoleacetic acid (VELIKY *et al.*, 1969), suggesting that the enzyme concentration may be correlated with tissue organization (SIMOLA, 1972b). The production of the paniculide sesquiterpene lactones is not significantly affected by the auxin level (BUTCHER, 1971).

2.2.3 Chemistry

Since the introduction of large-scale plant tissue culture fermentation (NICKELL, 1962), many improvements have been made in batch (VELIKY *et al.*, 1969), semi-continuous (KATO *et al.*, 1972; MANDELS *et al.*, 1968), and continuous fermentation systems (WILSON *et al.*, 1971). Nevertheless, it is extremely difficult to know if product production is affected more by the genetic or the chemical pressures resulting from the medium and its environment.

The medium composition may affect both growth and product production. It is well established that phosphates stimulate cell growth (KATO *et al.*, 1972). However, it is not yet well established how to initiate active photosynthesis in a tissue (PEAUD-LENOËL, 1974) so that it no longer requires a carbohydrate substrate. Although molasses was not satisfactory, many industrial cottonseed, meat, milk, and soy nitrogen supplements were satisfactory for dioscorea tissue growth (MEHTA *et al.*, 1972). Surface tension agents are often added to media for foam control, but their immediate and long-term effects on product production and release need to be studied more thoroughly. A number of nitrite, nitrate, and ammonia nutritional uptake studies are reported (DOUGALL, 1972; VELIKY and ROSE, 1973), and there is no doubt that the nitrogen uptake is affected by pH (ROSE and MARTIN, 1974). In some cases, nitrogen uptake has been correlated with tissue growth (HAHLBROCK, 1974) and product production (KONOSHIMA *et al.*, 1970).

Environmental factors such as light, temperature, length of fermentation growth cycle, and the gas phase may significantly affect the behavior of a tissue culture. Selective wave bands and/or the intensity of light will stimulate anthocyanin (CONSTABEL *et al.*, 1973), chlorophyll (STABA, 1969), flavonoids and lignification (BRUNET and IBRAHIM, 1973), phenylalanine ammonia lysate (HAHLBROCK *et*

al., 1971) and volatile oil synthesis (CORDUAN and REINHARD, 1972), and inhibit 1–4 naphthoquinone pigment synthesis (TABATA and MIZUKAMI, 1974). The fermentation temperature will affect the cell doubling time in a growth cycle (DOUGALL, 1972), and may induce tissue habituation. The cell doubling time is now known to be most often between one and four days (DOUGALL, 1972). Products such as phenylalanine ammonia lysate (MATSUMOTO *et al.*, 1973), tannins (CONSTABEL *et al.*, 1974), and visnagin (CHEN *et al.*, 1969) are induced early in a growth cycle. Lastly, the concentration of dissolved carbon dioxide may affect cell permeability (DOREE *et al.*, 1974), and that of oxygen may affect the differentiation process (KESSELL and GOODWIN, 1974).

3. Products

3.1 Tropane Alkaloids

Plants normally contain from 100–1000 mg-% dry weight total tropane alkaloids. Callus cultures contain approximately 1–35 mg-% (HIRAOKA *et al.*, 1973; KONOSHIMA *et al.*, 1970; SAIRAM and KHANNA, 1971); 50–100 mg-% (CHAN and STABA, 1965; NETIEN and LACHARME, 1965; KONOSHIMA *et al.*, 1967; WEST and MIKA, 1957); and root organ cultures from 500–1000 mg-% dry weight total alkaloids (MITRA, 1972; VAN HAGA, 1954). Minor alkaloids (STABA and JINDRA, 1968) and enzymes (JINDRA and STABA, 1968) have been identified and studied in *Datura stramonium* tissue cultures.

The tropane alkaloids scopolamine (hyoscine) and L-hyoscyamine (DL-atropine) result from the fusion of the base moiety tropine with the acid moiety tropic acid. This fusion may be accomplished by either *nicotiana* or *datura* tissue cultures (STOHS, 1969), and appear to favor the production of scopolamine (CHAN and STABA, 1965; STOHS, 1969). Tropine is biosynthesized from ornithine and tropic acid from phenylalanine.

The precursor ornithine appears to slightly increase alkaloid production (CHAN and STABA, 1965; KONOSHIMA *et al.*, 1970), but less than that by tropine. It is known that datura root organ cultures convert tropine to acetyltropine (ROMEIKE and AURICH, 1968), and it is suggested that undifferentiated cultures react similarly as its phenylalanine conversion to tropic acid is repressed (HIRAOKA *et al.*, 1973). Tropic acid does exert an effect on the organogenesis of belladonna cell suspensions (THOMAS and STREET, 1970). Both phenylalanine (CHAN and STABA, 1965; SAIRAM and KHANNA, 1971) and tyrosine (SAIRAM and KHANNA, 1971) are reported to increase the total tropane alkaloid content of tissue cultures.

3.2 Ginsenosides

Korean ginseng *(Panax ginseng)* plants contain similar triterpenoid ginsenosides as American ginseng *(Panax quinquefolium)* plants (KIM *et al.*, 1972). Undifferentiated Korean (FURUYA *et al.*, 1973) and undifferentiated or differentiated Ameri-

can ginseng tissue cultures (JHANG *et al.*, 1974) contain these ginsenosides in a concentration approximating that of the plant (0.4% purified saponins).

In 1967 the tissue cultures of ginseng were raised (BUTENKO, 1967; SLEPYAN *et al.*, 1967), and in 1968 fatigue suppressing tests upon rats with ginseng tissue culture extracts were performed (SLEPYAN, 1968). The Russian workers have also studied morphologic (BUTENKO *et al.*, 1968; SLEPYAN, 1971), physiologic (PISETSKAYA, 1970; ZHOLEKEVICH *et al.*, 1971), and genetic (BUTENKO *et al.*, 1974) aspects of ginseng tissue culture. The Koreans and the Japanese workers have also studied the physiology (KITA and SUGII, 1969), pharmacology, and chemistry (FURUYA *et al.*, 1970) of ginseng tissue culture. FURUYA and ISHII (1973) received a patent to manufacture the ginsenosides from tissue cultures, and METZ and LANG (1966) from root organ cultures.

3.3 Diosgenin

Diosgenin is industrially important as it is either microbially or chemically converted to medicinally useful steroids. It is principally obtained from the underground portions of various dioscorea plant species in which it is present in an approximate concentration of 4–5% dry weight (range 0–14%). Diosgenin is also reported in *Asparagus*, *Balanites*, *Costis*, *Momordica*, *Paris*, *Solanum*, and *Trigonella* species, and related steroids in *Agave*, *Lycopersicon*, *Solanum*, and *Yucca*.

As early as 1959 agave was studied as a tissue culture (WEINSTEIN *et al.*, 1959), but neither it nor dioscorea was reported by 1966 to contain industrially useful steroids (EHRHARDT *et al.*, 1966). Gitogenin was isolated and manogenin identified as present in *Yucca glauca* tissue cultures (STOHS and ROSENBERG, 1974). VAGUJFALVI studied dioscorea tissue cultures in 1967, and in 1971 reported diosgenin present in a 0.01–0.1% dry weight concentration in *solanum* callus cultures (VAGUJFALVI *et al.*, 1971). Trigonella tissue cultures contain trace amounts of diosgenin (STEVENS and HARDMAN, 1974), or as high as 1.8% diosgenin (KHANNA and JAIN, 1973). *Momordica charantia* suspension cultures contain 1.4% dry weight of diosgenin (KHANNA and MOHAN, 1973). One should keep in mind that diosgenin exists as the glycoside dioscin, and that it can also be dehydrated during its extraction with acids.

Many dioscorea species have been studied as tissue cultures. The Japanese workers have published extensively about *Dioscorea tokoro* tissue cultures from which they identified prototokoronin (TOMITA and UOMORI, 1974), tokorogenin, diosgenin, and other steroids (TOMITA *et al.*, 1970; TOMITA and UOMORI, 1971). Tissue cultures of *D. sylvatica* may contain as much as 1.2% dry weight diosgenin (KAUL, 1969), although most dioscorea species as tissue cultures contain between 5 and 300 mg-% dry weight diosgenin (KAUL, 1969; MEHTA and STABA, 1970).

The diosgenin content of *Dioscorea deltoidea* tissue cultures varies from less than 300 mg-% (MEHTA and STABA, 1970), 300–500 mg-% (ABROSHNIKOVA *et al.*, 1971; ABROSHNIKOVA and PANINA, 1972), approximately 1.0% (KAUL and STABA, 1968; KAUL, 1969), to 1.6% (KAUL *et al.*, 1969). Tissue cultures incorporate labeled cholesterol (STOHS *et al.*, 1969), sitosterol (STOHS *et al.*, 1974), and related steroids (TOMITA and UOMORI, 1971, 1974) into diosgenin.

3.4 Cardenolides

Many investigators have examined *Ammi*, *Apocynum*, *Cheiranthus*, *Digitalis*, *Oleander*, *Periploca*, *Urginea*, *Vincetoxicum* and other species of plants as tissue cultures for the presence of cardenolides (CAREW and STABA, 1965; STABA, 1969). As far as the author is aware a cardenolide has not yet been crystallized from tissue culture. However, positive Baljet substances tentatively identified as *Digitalinum verum* and verodoxin are in *Digitalinum mertonensis* tissue cultures (MEDORA *et al.*, 1967a, b); Kedde, Keller-Kliani, Legal and/or Liebermann positive substances in vincetoxicum (NETIEN and LACHARME, 1965) and digitalis tissue cultures (STABA *et al.*, 1965); pink rather than violet reacting Raymond substances and the glycoside sugar cymarose in apocynum tissue cultures (HARRIS *et al.*, 1964; LEE *et al.*, 1972); and positive reacting cardenolide substances in periploca (NETIEN *et al.*, 1967). The total concentration of the Kedde-positive substances is low, approximately 2–20 mg-% dry weight (STABA, 1969), and they have been stated to be anthraquinone pigments (FURUYA *et al.*, 1972). GRAVES and SMITH (1967) reported cardenolides to be absent from tissue cultures of various digitalis species. *Digitalis* tissue culture extracts produce a cardiac reaction in guinea pigs (MEDORA *et al.*, 1967), rabbits (KAUL *et al.*, 1967), and other animals (PETIARD and DEMARLY, 1972). *Digitalis* tissue cultures can also produce glycosides that are not cardenolides (PILGRIM, 1970).

3.5 Biotransformations

Compounds added to media as precursors (DOBBERSTEIN and STABA, 1969; CAREW and KRUEGER, 1974) or to be transformed (BODER *et al.*, 1964) to economically important alkaloids, or other compounds (BARZ *et al.*, 1974) have been studied by both growing cultures (KATO *et al.*, 1972) and their cellular fractions (BENVENISTE *et al.*, 1967).

Dioscorea deltoidea tissue culture can utilize cholesterol to produce increased amounts of diosgenin (KAUL *et al.*, 1969), to convert progesterone to a conjugate form of both 5α-pregnan-3-ol-20-one and 5α-pregnan-3B, 20B-diol (STOHS and EL-OLEMY, 1972a), and to convert the androgen, 4-androsten-3, 17-dione, to 5α-androstan-3B-ol-17-one and 5α-androstan-3B, 17B-diol but not diosgenin (STOHS and EL-OLEMY, 1972b). STOHS and his coworkers have also demonstrated that the microsomes of *D. deltoidea* will convert progesterone to 5αpregnan-3, 20 dione.

Digitoxigenin was converted to non-glycosidic compounds by both digitalis and mint tissue cultures (STOHS and STABA, 1965). However, *Digitalis purpurea* cultures did specifically convert digitoxin to purpurea glycoside A, B, gitoxin, and reduced progesterone (FURUYA *et al.*, 1973). Progesterone and pregnenolone can be transformed by a number of tissue cultures, but could not be converted to cardenolides (GRAVES and SMITH, 1967; FURUYA *et al.*, 1973). Tissue cultures of *D. mertonensis* will utilize cholesterol to produce cardenolides (MEDORA *et al.*, 1967). Selected *Digitalis lanata* cultures can hydroxylate digitoxin at the 12 position, add glucose to the carbohydrate side chain, and acetylate digitoxose (REINHARD, 1974).

4. Conclusion

One cannot be certain where or when products will be successfully produced from plant tissue cultures for commercial purposes. It may be from a product not here identified, or it may be a product from the sulfur-containing volatiles (SELBY and COLLIN, 1974), amino acids (MISAWA *et al.*, 1974), plant allergens (SHAFIEE and STABA, 1973), enzymes (KATO *et al.*, 1973), or hormones (KHANNA *et al.*, 1974). Bioassays have significantly assisted us to establish whether tissue cultures contain antibiotics, anti-fatigue compounds, cardenolides, hormones, insecticides, tumor inhibitors, etc. Dramatic progress has also been made with plant cultures to improve plant strains (NICKELL and HEINZ 1973), to study comparmentalization (NETTLESHIP and SLAYTOR, 1974), and to study the site of cellular biosynthesis by compound autofluorescence (RIVIER *et al.*, 1974) and perhaps with fluorescent and/or labeled specific antibodies. Commercial success for product biosynthesis from plant tissue cultures should be closer at hand than ever!

References see page 703.

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Chapter VII Miscellaneous

1. Isozymes in Plant Tissue Culture

J. G. SCANDALIOS and J. C. SORENSON

1. Introduction

It is now an established fact that most enzymes exist in multiple molecular forms (isozymes). It is further acknowledged that a large amount of variation detected in isozyme studies is due to genetic bases, thus rendering isozymes as useful "markers" in a number of biological investigations. The variant molecules provide a natural label which makes them readily detectable in small quantities of biological material. In most cases the catalytic activity of the enzyme variants is unaltered and thus their presence does not affect the biological system in any adverse way. Once variants are genetically defined, they can be used as critical markers in the analysis of gene function and metabolic regulation in growing and differentiating cells.

Genetically defined variants of isozymes have been applied as research tools in such studies as: (1) evolutionary relationships at the molecular level; (2) enzyme structure and function; (3) determination of polymorphic loci in populations; (4) metabolism; (5) nucleo-cytoplasmic relationships; etc. (see MARKERT, 1975). However, isozymes have not yet been fully exploited as efficient and effective markers in tissue culture research and somatic cell hybridization, particularly in plant systems. Although some conventional markers are available and have been used in studies with cultured cells, in many higher plants they are not the best markers. The deliberate incorporation of nutritional deficiencies into standard culture procedures is inconsistent with the current emphasis on agronomic applicability. Where the parasexual cycle is attempted, the expression of the marker system should be discernible at a fairly early stage (unlike most morphological markers that can be detected only after the differentiation of an organ or tissue). In this respect, isozymes constitute ideal markers for tissue and somatic cell genetic studies due to: (1) the ease of detection; (2) the abundance of naturally occurring variants in most plant populations obviating the necessity for mutant induction (ALLARD and KAHLER, 1971, estimate that 31–54% of the enzyme loci in higher plants are naturally polymorphic); (3) applicability to small amounts of tissue and crude extracts; and (4) the fact that in most cases, the marker is expressed in the "undifferentiated" state of a cell culture. Phenotypic markers such as morphology, color, growth, etc., are usually several steps removed from the gene. Isozymes, in contrast, are direct gene products and are, therefore, less susceptible to phenocopy responses and modification by environmental variables.

In this article, we will attempt to discuss some aspects of tissue culture which can be facilitated by the utilization of isozymes. Following a brief overview of some basic isozyme methodology, we intend to delineate and discuss the various

applications of isozymes in tissue culture research. These will include areas of practical importance such as monitoring the stability of cells in culture, detection of hybrid cells, as well as such potential areas as assessment of possible "developmental" stages in cultured cells, studies of gene regulation, and measuring the genetic contribution of each parental cell type in hybrid cells and in parasexually induced hybrid plants.

2. Isozymes: Definition, Resolution, Classification

2.1 Definition

Isozymes are multiple molecular forms of an enzyme with similar or identical substrate specificities occurring within the same organism (MARKERT and MØLLER, 1959). Enzymes with very broad substrate specificities were not included in the above definition. The phenomenon of enzyme multiplicity had been known in a few cases for many years, but it was not until the development of the zymogram technique by HUNTER and MARKERT (1957) that the occurrence of isozymes came under extensive investigation. This technique involves electrophoretic separation, usually of crude tissue extract, followed by demonstration of zones of enzyme activity using specific histochemical staining procedures applied directly to the electrophoretic medium (e.g. agar, starch, acrylamide). The facility of the zymogram method had made possible the rapid screening of a large number of enzymes in relatively small amounts of tissue extracts. However, although powerful, this technique allows for the rosolution of isozymes mainly on the basis of charge and size differences; it does not discriminate mutants that may have similar electrophoretic mobilities, but may differ in physicochemical properties. Amino acid substitutions which impart no changes in the net charge of the enzyme may affect other properties of the enzyme (see MARKERT, 1975). In more recent years a number of laboratories have been involved in employing other tools of protein chemistry, both new and conventional, to examine the latter parameters. Consequently, these investigations of multiple enzyme forms have led to a need for more precisely defined or categorized isozymes according to the level at which they have been investigated.

2.2 Resolution

As previously mentioned, enzyme heterogeneity has been resolved by the popular and convenient technique of gel electrophoresis in conjunction with the zymogram technique. The great advantage of this approach is that it can be carried out on crude tissue extracts. Since enzyme purification is not a necessary part of the technique, such studies are within the capabilities of many laboratories. To date, the various electrophoretic procedures remain the technique of choice, and are still the most convenient and probably the most precise tools for initial investigations of isozymes. For more refined investigations, more use is made of other techniques such as electrofocusing, gel filtration, ion exchange chromatography,

sedimentation, and heat or inhibitor sensitivity. MARKERT (1975) has recently edited an excellent compilation of current isozyme methodology and experimental approaches in isozyme investigations.

2.3 Classification

Since the term isozyme was intended to be, and is, an operational definition, the need for more precise distinction of isozyme types has arisen with the depth of investigation of each system. Though any classification of isozymes is bound to be inadequate, a simple system resulted from discussions at the Isozyme Conference in Williamsburg, Virginia in 1965, and subsequently described by SHAW (1969).

This classification separates isozymes into two major categories: (1) those that are distinctly different molecules (polypeptides) and which are presumably encoded at different genetic sites; and (2) those which result from secondary modifications in the structure of a single polypeptide species and may, in many cases, be *in vitro* artifacts. It has also been proposed (MARKERT, 1968) that the term isozyme may be modified by such adjectives as allelic, nonallelic, homomultimeric, or heteromultimeric (depending on the subunit composition), hybrid, conformational, conjugated, etc. to reflect more precisely our knowledge of the particular isozyme system.

Isozymes may arise by a variety of chemical or physical means, such as: binding of a single polypeptide to varying numbers of coenzyme molecules (URSPRUNG and CARLIN, 1968) or to other prosthetic groups (e.g. divalent cations, carbohydrates, AMP, etc.); by conjugation or deletion of molecules with reactive groups such as amino, carboxyl, or hydroxyl groups of amino acid residues. Multiple forms of some enzymes may, for example, be generated by preparative procedures or during storage. Horseradish peroxidase isozymes may, for example, be generated and interconverted by manipulation of pH (LIU, 1971). It is for these reasons that genetically and biochemically well-defined isozymes are highly preferable in studies of tissue culture and cell hybridization. Plant isozyme systems which have been genetically defined are listed in Table 1.

3. Some Experimental Procedures

Following are some of the isozyme methods used in our laboratory. All of the procedures given work well with a wide variety of plant systems. Additional staining techniques may be found in BREWER (1970).

3.1 Preparation of Starch Gels

Sufficient hydrolyzed potato starch is suspended in an appropriate buffer (Table 2) to make a 12% gel. The suspension is then heated with constant agitation until it is just at the boiling point. The liquid is then aspirated for approxi-

Table 1. Genetically defined isozyme systems in plants

Plant	Enzyme	No. Isozymes	No. Defined Loci	Reference
Barley	α-Amylase	5	1	FRYDENBERG and NIELSEN (1965)
Maize	Acid phosphatase	10	3	EFRON (1970)
	Alcohol dehydrogenase	1–4	2	FELDER and SCANDALIOS (1971) SCANDALIOS (1969a), SCANDALIOS and FELDER (1971)
	α-Amylase	2–3	1	CHAO (1970) CHAO and SCANDALIOS (1969, 1971)
	β-Amylase	4	1	CHAO (1970) CHAO and SCANDALIOS (1969, 1971)
	Catalase	1–5	2	BECKMAN *et al.* (1964a) QUAIL and SCANDALIOS (1971) SCANDALIOS (1968) SCANDALIOS *et al.* (1972)
	Catechol oxidase	2	1	PRYOR and SCHWARTZ (1973)
	Endopeptidase	2	1	MELVILLE and SCANDALIOS (1972) NIELSEN and SCANDALIOS (1974)
	Esterase		3	SCHWARTZ (1960)
	Glutamate Dehydrogenase	3	1	PRYOR (1974)
	GOT (Aspartate Aminotransferase	7	1	SCANDALIOS *et al.* (in prep.)
	Leucine amino peptidase	4	4	BECKMAN *et al.* (1964b) SCANDALIOS (1969)
	Malate dehydrogenase	7–9	4	YANG and SCANDALIOS (1974, and in prep.)
	Peroxidase	12	1	SCANDALIOS (1969) HAMILL (1970)
Pea	Aminopeptidase (LAP)	3	3	SCANDALIOS and CAMPEAU (1972) SCANDALIOS and ESPIRITU (1969)
Wheat	Alcohol dehydrogenase	3	1	HART (1970, 1971)
	α-Amylase	16	2	DAUSSANT and COURVAZIER (1970) NISHIKAWA and NOBUHARA (1971) DAUSSANT and RENARD (1972)

Table 2. Some basic electrophoretic techniques for plant materials

Enzyme	Gel buffer	Electrode buffer	Stain components	
Aminopeptidase	Buffer C	Buffer C	L-Leucyl β-naphthylamide	HCl
			Black k Salt	10 mg
			Tris maleate	20 mg
			buffer (0.2 M, pH 6.5)	100 ml
Acid phosphatase	1 part Buffer D 9 parts Buffer B	Buffer D	α-Naphthyl acid phosphate	100 mg
			Fast garnet GBC	100 ml
			10% aqueous $MgCl_2$	10 drops
			Na-acetate buffer (0.2 *M*, pH 4.0)	100 ml
Alkaline phosphatase	1 part Buffer D 9 parts Buffer B	Buffer D	α-Naphthyl acid phosphate (NaSalt)	100 mg
			Fast blue RR salt	100 mg
			10% Aq. $MgCl_2$	10 drops
			10% Aq. $MnCl_2$	10 drops

Table 2 (continued)

Enzyme	Gel buffer	Electrode buffer	Stain components	
Alcohol dehydrogenase	1 part Buffer A 60 parts H_2O	1 part Buffer A 20 parts H_2O	0.002 *M* KCN	2 ml
			0.01 *M* NAD	2 ml
			0.01 *M* PMS	2 ml
			Ethanol (100%)	1 ml
			NBT	50 mg
			Tris-HCl Buffer (0.015 *M* pH-8.0)	180 ml
Catalase	1 part Buffer A 60 parts H_2O	1 part Buffer A 20 parts H_2O	Flood gel surface with 0.5% H_2O_2 for approximately 1 min, rinse with distilled H_2O, and flood surface with 1.5% KI solution acidified with 12 drops glacial acetic acid/30 ml	
Esterase			α-Naphthyl acetate (1% in 1:1 acetone-H_2O)	2 ml
			Fast blue RR salt	40 mg
			0.1 *M* K-phosphate Buffer pH	100 ml
Malate dehydrogenase	1 part Buffer A 60 parts H_2O	1 part Buffer A 20 parts H_2O	DL-Malic acid	1.38 g
			KCN (0.1 M)	0.4 ml
			NAD (0.01 M)	1 ml
			PMS	1.6 mg
			NBT	50 mg
			Tris-HCl (0.1 *M*, pH 8.0)	100 ml
Endopeptidase	Buffer C	Buffer C	α-N-Benzoyl-DL-arginine-β-naphthylamide HCl	10 mg
			Black K salt	20 mg
			Tris-maleate buffer (0.1 *M*, pH 6.5)	100 ml
GOT (Aspartate aminotransferase)	1 part Buffer D 9 parts Buffer B	Buffer D	Aspartic acid	130 mg
			α-Ketoglutarate	85 mg
			pyridoxal-5′-phosphate	1.5 mg
			Fast blue RR salt	180 mg
			0.1 M Phosphate buffer (pH 7.4)	100 ml
			Adjust pH of final mixture to 7.4 with 5N-NaOH.	

Buffer A. Tris-Citrate (1.0 *M*, pH 7.0)
A 1.0 *M* solution of Tris is titrated to pH 7.0 with solid citric acid.

Buffer B. Tris-Citrate (0.05 *M*, pH 8.25)
6.2 g Tris
1.6 g Citric acid (anhydrous)
H_2O to 2 l

Buffer C. Tris-Glycine (0.025 *M*, pH 8,7)
3.0 g Tris
14.4 g Glycine
H_2O to 1 l

Buffer D. *Lithium Borate* (1.0 *M* w/borate, pH 7.8)
0.7 g Lithium hydroxide (anhydrous)
11.9 g Boric acid (anhydrous)
H_2O to 1l

mately 30 sec for degassing and is poured into a lucite mold. The gel is allowed to solidify at room temperature for approximately 10 min, and is then refrigerated for several minutes prior to use.

3.2 Application of Sample

Samples may be applied to the gel in one of two ways. Extracts may be loaded directly into wells formed in the gel with a template prior to cooling, or they may be absorbed into small rectangular pieces of Whatman No. 1 filter paper and inserted into a slit cut into the gel (SCANDALIOS, 1969 b). We have found the latter method to be more convenient. Banding quality is often enhanced if the sample papers are removed after 45 min to 1 h of electrophoresis. As many as 30 samples may be applied to a single gel. Since optimal amounts of sample may vary widely from tissue to tissue, details of extract preparation etc. are best determined by trial and error for each particular system. Examples of techniques used in various plant systems may be found in the references in Table 3.

3.3 Electrophoresis

Gels are generally subjected to electrophoresis for 12–18 h (depending on the buffer used) at 7–10 V/cm in a refrigerated (4° C) cabinet. Following electrophoresis, the gels are sliced horizontally into 1.5 mm sheets and each sheet is placed in a shallow tray containing the appropriate histochemical stain (Table 2). Three sheets can generally be cut from a single gel. A large number of cell cultures can be studied in a relatively short time by application of many extracts to a single slab of electrophoretic medium. The slab technique described above, with samples applied across a straight row, permits precise comparison of migration rates so

Table 3. Isozyme systems in commonly cultured plants

Plant	Tissue	Enzyme	No. Isozymes	Reference
Brassica	Head	Malate dehydrogenase	2	RAGLAND *et al.* (1972)
		Thioglucosidase	4–5	HENDERSON and MCEWEN (1972)
Datura	various	Malate dehydrogenase	7	GANAPATHY and SCANDALIOS (1973)
	various	Peroxidase	3–9	CONKLIN and SMITH (1971) FERRI and GUZMAN (1970) GUZMAN *et al.* (1971)
Daucus	root	Invertase	2	RICARDO and REES (1970)
	suspension culture	Ribonuclease	2	WILSON (1971)
Dianthus	callus culture	Acid phosphatase	1–7	MCCOWN *et al.* (1970)
		Esterase	4–12	MCCOWN *et al.* (1970)
		Peroxidase	4–8	MCCOWN *et al.* (1970)

Table 3 (continued)

Plant	Tissue	Enzyme	No. Isozymes	Reference
Glycine		Lipoxygenase	2	CHRISTOPHER and AXELROD (1971) CHRISTOPHER *et al.* (1970) STEVENS *et al.* (1970)
		Peroxidase	8	HO and WEAVER (1970)
Hordeum	leaves	Acid phosphatase	4–5	SAKO and STAHMANN (1972)
	leaves	Acetyl esterase	9–11	SAKO and STAHMANN (1972)
	leaves	Alcohol dehydrogenase	3	SAKO and STAHMANN (1972)
	aleurone	α-Amylase	4	JACOBSEN *et al.* (1970)
	seeds		5	TANAKA and AKAZAWA (1970)
	aleurone	β-Amylase	4	JACOBSEN *et al.* (1970)
	seedling	Esterase	7–14	MARSHALL and ALLARD (1969)
	leaves	Glucokinase	6	SAKO and STAHMANN (1972)
	leaves	Glucose-P-isomerase	3–4	SAKO and STAHMANN (1972)
	leaves	Glutamate dehydrogenase	2	SAKO and STAHMANN (1972)
	seeds	Glycollate oxidase	2	DUFFUS (1970)
	leaves	Leucine aminopeptidase	3	SAKO and STAHMANN (1972)
	leaves	Malate dehydrogenase	13	SAKO and STAHMANN (1972)
	seeds	Malate dehydrogenase	2	DUFFUS (1970)
	leaves	Peroxidase	5–6	SAKO and STAHMANN (1972)
	leaves	6-phosphogluconate dehydrogenase	2	SAKO and STAHMANN (1972)
	leaves	Polyphenoloxidase	4–5	SAKO and STAHMANN (1972)
Lycopersicon	fruit	Pectinesterase	4	PRESSEY and AVANTS (1972)
	fruit	Peroxidase	2–9	FRENKEL (1972) GORDON and ALRIDGE (1971) KU *et al.* (1970)
Nicotiana	leaves	Esterase	2	REDDY and GARBER (1971)
	suspension culture	Malate dehydrogenase	4	DEJONG and OLSON (1972)
	various	Peroxidase	2–20	CHANT and BATES (1970) LEE (1971 a, b, c, 1972 a, b) LESHEM and GALSTON (1971) REDDY and GARBER (1971) RITZERT and TURIN (1970) SCHAFER *et al.* (1971) SHEEN and REBAGAY (1970) WOOD (1971)
	pith	Polyphenoloxidase	3–4	STAFFORD and GALSTON (1970)
	callus	Ribonuclease	4	WILSON (1971)
Oryza		Acid phosphatase	7	ENDO *et al.* (1971)
		α-Amylase	9	NISHIKAWA and NOBUHARA (1971)

Table 3 (continued)

Plant	Tissue	Enzyme	No. Isozymes	Reference
Oryza		β-Amylase	2	BAUN *et al.* (1970)
		Amylopectin debranching enzyme	2	BAUN *et al.* (1970)
		Peroxidase	3	ENDO (1971)
		Phosphorylase	3	GERBRANDY and VERLEUR (1971)
		Starch synthetase	2	TANAKA and AKAZAWA (1971)
Phaseolus		Chalcone-Flavone isomerase	2	HAHLBROCK *et al.* (1970)
	seedling	Chorismate mutase	2	GILCHRIST *et al.* (1972)
		Esterase	14	TSAI *et al.* (1970) VEERABHADRAPPA and MONTGOMERY (1971b)
	callus culture	Peroxidase	2	MISAWA and MARTIN (1972)
	various	Phosphorylase	1–5	GERBRANDY and VERLEUR (1971)
Pisum	leaves	Aldolase	2	ANDERSON and ADVANI (1970)
	seed	Esterase	7	VEERABHADRAPPA and MONTGOMERY (1971a)
	leaves	Glutamate dehydrogenase	4	KRETOVICH *et al.* (1971)
	leaves	Glycerate-3-phosphokinase	2	ANDERSON and ADVANI (1970)
	various	Leucine aminopeptidase	3	SCANDALIOS and CAMPEAU (1972) SCANDALIOS and ESPIRITU (1969)
	seed	Lipoxygenase	2	ERIKSSON and SVENSSON (1970)
	various	Peroxidase	3–5	CZAPSKI and ANTOSZEWSKI (1971) SANO (1970)
	leaves	Ribose-5-phosphate isomerase	2	ANDERSON (1971)
	leaves	Triose-phosphate-isomerase	2	ANDERSON and ADVANI (1970)
Sinapis	various	Peroxidase	3	BAJAJ *et al.* (1973)
Solanum	tuber	Acetyl CoA synthetase	5	HUANG and STUMPF (1970)
	tuber	Acid phosphatase	2	DESBOROUGH and PELOQUIN (1971)
	tuber	Apyrase	2	TRAVERSO-CORI *et al.* (1970)
	tuber	Invertase	5	SASAKI *et al.* (1971)
	various	Phosphorylase	1–9	GERBRANDY and VERLEUR (1971)
Vicia	various	Phosphorylase	4–8	BAUN *et al.* (1970)
Zea	See Table 1			

that slight differences are readily detectable. Such comparisons are difficult to make with single sample disc gels. In addition, starch slabs may be sliced into several layers, and each stained for a different enzyme activity. This allows for direct comparison of the relative migration rates of several enzymes from a single electrophoretic run. In addition, with enzymes which can utilize a series of substrates (e.g. aminopeptidases), it is possible to assess relative substrate specificities of isozymes using a single sample.

4. Cell Recognition

Isozyme variants as a class are more numerous than any other genetic markers and possess a number of advantages for tissue culture and somatic cell genetic analysis. Numerous isozyme systems have been studied in plants which are frequently studied in culture (Table 3), and one might expect the isozyme complement of cultured cells to be similar to that of intact plants in many cases. A prerequisite, however, would be to screen cultures in order to establish such homologies. *Datura innoxia* and *Datura metel* have been shown to have distinctly different malate dehydrogenase (MDH) isozyme patterns (GANAPATHY and SCANDALIOS, 1973). The appearance of a "hybrid" pattern in a mixture of extracts from the two species suggests that this enzyme may be a useful marker system for distinguishing hybrid protoplasts from parental types in culture. Peroxidase isozymes have been used to identify sexually produced interspecific hybrids in *Nicotiana* (HOESS *et al.*, 1974), and CARLSON *et al.* (1972) have used the same enzyme to identify parasexually produced hybrids in the same genus.

In cases where the zymogram pattern of a hybrid cell is a simple additive composite of the two parental patterns, it is difficult to distinguish between true hybrid cells and mixtures of parental cells. This difficulty may be overcome by the use of isozyme systems which form heteropolymeric enzymes when variant en-

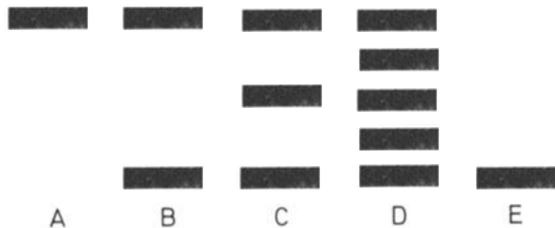

Fig. 1A-E. Hybrid isozyme patterns. (A) and (E) represent two parental types with distinctly different isozyme forms. (B) through (D) represent possible isozyme patterns in a hybrid between (A) and (E). Pattern (B) represents the expected pattern if the enzyme were a monomer (and no hybrid molecules could be formed), or if the enzyme were a multimer with no subunit interactions. Pattern (C) represents the expected zymogram if the enzyme were a dimer. The middle band represents a hybrid enzyme with one (A) and one (E) subunit. Pattern (D) represents a tetramer. The three middle bands represent hybrid molecules with various combinations of (A) and (E) subunits

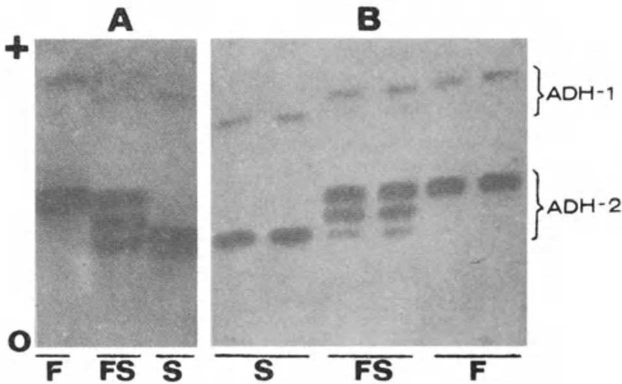

Fig. 2A and B. Zymogram of maize alcohol dehydrogenase showing gene dosage effects on the ADH-2 isozymes. (A) extracts from scutella (2n) of single kernels of the fast *F* and slow *S* ADH-2 variants and their F_1 hybrid *FS*. (B) extracts from liquid endosperm (3n) of the same kernels where the maternal parent was the ADH-2F variant. Note the gene dosage (as reflected by staining intensity or activity of the bands) in the endosperm where the activity distribution is 4FF:4FS:1SS as compared to the diploid scutellum where it is 1FF:2FS:1SS. In maize, ADH is a dimer. 0 = point of sample insertion. Migration is anodal at pH 7.4. Zymogram also shows ADH-1 which is determined by a distinct, but closely linked gene

zymes are expressed within the same cell (Fig. 1). This approach has been used successfully in cultured animal cells (RUDDLE, 1969).

Another advantage of the use of isozymes as marker systems for parasexually produced hybrids is that this technique allows one to estimate the relative contribution of each parental genome to the hybrid pattern. In triploid maize endosperm for example, where the maternal parent contributes twice the genetic information of the paternal parent, the gene dosage effect is readily apparent on the zymogram pattern (Fig. 2).

5. Physiological Studies

The primary use of isozymes in plant tissue culture to date has been in relation to physiological studies. Isozyme patterns frequently vary in intact plants as a function of various physiological and developmental states (reviewed by SCANDALIOS, 1974), and one might expect similar responses in cultured cells. LEE (1971a, b, c, 1972a, b) has shown this to be the case for peroxidases ("IAA oxidase") in *Nicotiana* in a series of studies using exogenously supplied hormones, and LAVEE and GALSTON (1968) have found similar effects in *Pelargonium* cultures. In *Nicotiana* the pattern of peroxidase expression is influenced both quantitatively and qualitatively. Indoleacetic acid at optimum concentrations causes the appearance of two new peroxidase isozymes. Levels of the hormone sufficient to inhibit growth cause an increase in the activity of the constitutive isozymes, but will not "induce" the

new forms. The same effect is observed with 2,4-D. Kinetin and zeatin (a mitogen) cause the appearance of the new isozyme forms, as does gibberellin GA_3 in the presence of IAA. In all cases the appearance of the additional isozymes is inhibited by cycloheximide and actinomycin D, suggesting that RNA and protein synthesis may be necessary for the increase. Such inhibition does not, however, absolutely prove this to be the case, nor is it sufficient proof that the enzyme is synthesized de novo. LEE (1971b) further speculates that GA acts in the system at the level of RNA synthesis, a conclusion which is in no way warranted by the evidence he provides.

Since peroxidase is probably involved in IAA metabolism, the study of the enzyme is of interest in growth and differentiation processes in culture. The system demonstrates dramatically, however, the need for using biochemically and genetically defined systems for such studies. In the case of peroxidases it is impossible to interpret the appearance of new bands in terms of physiological significance. Peroxidases almost certainly perform a number of diverse functions in the cell in addition to IAA oxidation (e.g. ligninification; HARKIN and OBST, 1973), and the new forms may be totally unrelated to the process in question. Without adequate characterization, the possibility that the "induced" forms may be artifacts also cannot be discounted.

Similar criticisms may be made with regard to the studies of McCOWN *et al.* (1970) on several enzymes in *Dianthus* culture. Although definite qualitative changes in the isozyme patterns of peroxidase, esterase, and acid phosphatase were observed in response to varying light and temperature conditions, the significance of these changes cannot be assessed without further characterization of the system. All three of these enzymes are thought to exhibit broad substrate specificities, and the isozymes probably have diverse physiological functions.

Variations of light and temperature have also been shown to influence the zymogram patterns of MDH in *Nicotiana* (DEJONG and OLSON, 1972), and the extent to which the enzyme has been characterized in other systems will undoubtedly aid the further characterization and interpretation in this system.

The regeneration of whole plants from cultured cells has been an area of intense investigation. Isozymes may provide a unique tool in such studies. It has been well established that many plants undergo striking changes in isozyme expression at or about the time of seed germination (SCANDALIOS, 1974). Since such changes accompany rapid growth and differentiation in intact seeds, isozymes may provide a sensitive marker system for genetic and physiological changes preceding differentiation in cultured cells as well. Studies along these lines are currently being undertaken with chromosomal proteins in carrot culture (GREGOR *et al.*, 1974), and in *Sinapis* with peroxidases (BAJAJ *et al.*, 1973). Studies with peroxidases in carrot and aspen cultures (WOCHOK and BURLESON, 1974; WOLTER and GORDON, 1975) also indicate that isozymes may be sensitive markers of the differentiation process. The lack of characterization of the systems is perhaps somewhat less consequential in studies such as these since their primary use is as a marker, and physiological consequence is not of primary concern at this stage of the investigations. In addition, a number of investigators have measured enzyme activities as indicators of differentiation in cultured cells (e.g. Simola and SOPANEN, 1970). This aspect has been reviewed by BAJAJ and BOPP (1971).

6. Conclusions

Isozymes have a number of inherent advantages over morphological and nutritional markers in tissue culture studies. The isozyme technique requires small amounts of material, can be applied to undifferentiated cells in culture as well as to intact or regenerated plants, necessitates no severe manipulation of the system, allows for the assessment of relative genome contributions in hybrid cells or tissues, and in many instances provides a very sensitive marker system for the physiological state of a cell or tissue. In spite of these advantages, isozymes have not been fully exploited in most plant tissue culture studies.

Although literature pertaining directly to isozymes in plant tissue culture is sparse, it is hoped that the approaches outlined in this article may provide a stimulus for such studies. Hopefully the tables and techniques presented will prove useful in bridging the gap between the abundance of isozyme information in intact plant systems and the potential use of isozymes in plant tissue culture studies.

References see page 778.

2. Radiation Biology of Cultured Plant Cells

G. P. HOWLAND and R. W. HART [1]

1. Introduction

Plant tissues can be isolated and cultured in vitro by the application of relatively simple techniques and their use offers great advantages: (1) to the biochemist, who can manipulate the cellular environment with added effectors (e.g. hormones, metabolic precursors, inhibitors) while avoiding contributions by contaminating microorganisms; (2) to the morphologist, who can explore the developmental potential of single somatic cells; (3) to the geneticist, who can treat millions of isolated cells as individual mutable units and select for a rare variant in a few petri dishes; and (4) to the cell biologist, who can study the basic physiology of a population of essentially identical cells. The potentials of these and other approaches have been presented elsewhere in this volume. This discussion is focused on the radiation [2] biology of cultured plant cells. Where information is incomplete or absent for cultured plant cells, we have drawn upon the microbial and animal cell literature for reference.

In animals the lethal effects of ionizing radiation are reflected in killing of specific cell types (e.g. crypt cells of the intestine and the stem cells of the bone marrow), and this determines the survival frequency of the irradiated animal population (HALL, 1973). Nevertheless, studies on cultured mammalian cells have contributed much to our present understanding of the biochemical, biophysical, and genetic aspects of radiation damage and recovery in animals (see ELKIND and WHITMORE, 1967; CLEAVER, 1974). In contrast, irradiation of plants, while producing some differential cell killing, does not appear to induce death of the organism by affecting a single cell type. Thus, studies with plant cells in culture may reflect more the effects of penetrating radiation on the organism as a whole than those with animal cell cultures.

The effects of ionizing radiation have been studied in many plant species (e.g. Sparrow *et al.*, 1958, 1965; DAVIDSON, 1960; ROMANI, 1966; HÄBER, 1968, 1972; VERMA, 1974), but the complexity of the intact organism has left many basic questions unanswered regarding aspects of the molecular and cellular recovery processes following damaging doses of ionizing radiations.

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[2] Radiation, as used in this article, refers to the short-wavelength electromagnetic radiations: ultraviolet light (UV) and ionizing radiations (X-rays and γ-rays). Unless otherwise stated, UV is used to indicate far-UV radiation (primarily 254 nm) obtained from "germicidal" lamps.

Earlier work on radiation effects in cultured plant cells (e.g. KLEIN and VOGEL, 1956; MELCHERS and BERGMANN, 1959; JONARD, 1959; KLEIN, 1963, 1968; HOLSTEN *et al.*, 1965; VENKETESWARAN and PARTANEN, 1966; ERIKSSON, 1967a, CRUZ and HILDEBRANDT, 1968) focused primarily on the evaluation of growth inhibitions resulting from UV or ionizing radiation exposure. Questions relating to molecular radiation effects in cultured plant cells have been reported more recently (e.g. TROSKO and MANSOUR, 1968, 1969a, b; BAJAJ, 1973; VERMA and VAN HUYSTEE, 1971b; OHYAMA *et al.*, 1974; MURPHY *et al.*, 1975; HOWLAND, 1975; HOWLAND *et al.*, 1975). We will consider these aspects in detail (see Sects. 4.1, 4.2, and 4.5). We will also discuss the various criteria which have been employed in evaluating the radiation sensitivity of cultured cells, and will emphasize the need to measure single-cell colony formation in radiation survival experiments (see Sect. 4.6).

Data on the UV sensitivity of plant cells is needed especially in view of the predicted effect of current technology in partially destroying the UV-filtering ozone layer in the stratosphere (e.g. JOHNSON, 1971; ROWLAND, 1974). Plants are naturally exposed to significant quantities of UV in sunlight in the range of 280–315 nm ("UV-B"), and the damaging effects of solar UV have been demonstrated in plants grown under conditions which excluded the longer-wavelength radiation responsible for photoreactivation of UV damage (CALDWELL, 1971). Some important questions which can be answered using cultured cells are: how will natural and cultivated plants respond to increased amounts of solar UV? Can plant cells recover from UV damage? What is the extent of species variation in UV recovery capacity? Are plants now operating close to the limit of their recovery capacity? Do plants accumulate UV-induced damage in their genetic material?

2. Irradiation of Cells and Protoplasts

Plant cell cultures, while less studied than animal cells, offer some unique advantages. Especially significant are the availability of completely defined media for the growth of plant cells, haploid cell cultures, and technique which allow the recovery of the whole plant from tissue cultures. Cultured plant cells provide excellent material for radiation studies since the use of single cells obtained from liquid suspension cultures or of isolated protoplasts allows quantitative radiation dosimetry and analysis of single-cell survivals. In addition, the absence of a cell wall makes protoplasts ideal for the isolation and analysis of very high molecular weight DNA after radiation treatment (HOWLAND, 1975; HOWLAND *et al.*, 1975). Nucleic acids can be labeled with radioisotopes for studies of radiation-induced damage and repair without interference from contaminating microorganisms often encountered in whole-plant or organ experiments (LONBERG-HOLM, 1967). Caution must be exercised, however, to avoid or inhibit catabolism of the radioactive pyrimidines supplied to the plant cells (HOWLAND and YETTE, 1975). When degradation is serious, labelling can be very inefficient and sometimes nonspecific (TAKATS and SMELLIE, 1963).

A unique advantage of plant cells is the availability of haploid material and aneuploid stocks of several different species. By use of monosomic and trisomic lines for radiation studies, it should be possible to test directly the contributions of separate chromosomes to radiation recovery processes and thus to map genetically the location of specific recovery functions (CARLSON, 1972). The utility of haploid cells for mutation studies has been discussed by others (see CHALEFF and CARLSON, 1974; and Chap. II of this Vol.). The two most useful aspects of haploid mutagenesis are the immediate expression of recessive mutations and the ease with which homozygous diploids can be obtained by colchicine-doubling of haploids. Since some cultured plant tissues can be induced to differentiate the intact plant (see Chap. I, 10 of this Vol.), the breeder can also conduct formal genetic analysis of mutants induced and isolated in vitro, with the possibility of bringing a new genotype into agricultural use (CARLSON, 1973).

These characteristics of cultured plant cells are especially appealing for basic research in genetics and development since there are no available mammalian cell systems which are either haploid or able to totally redifferentiate in vitro. Many problems in mutagenesis, gene expression, and differentiation can be readily studied with plant cell cultures.

However, there are still some significant limitations on the use of plant cultures for radiation studies. Single-cell preparations are essential for strictly quantitative assay of cell survival by cloning in petri dishes, but many plant suspension cultures do not yield adequate numbers of free cells which can be separated from cellular aggregates. As a convenient recourse, protoplasts can be enzymatically isolated in large quantities, thereby providing the required starting material for survival assays. The plating efficiency of most isolated plant cells and protoplasts tends to be rather poor, especially at low cell densities (below 10^4/ml) (NAGATA and TAKEBE, 1971; DOUGALL, 1973; ENGVILD, 1974; and Chap. IV, 1 of this Vol.). This density dependence can be partially overcome by the addition of "conditioned" medium (ERIKSSON, 1967a) or by a "feeder layer" of radiation-killed cells (RAVEH *et al.*, 1973). Recent work indicates that improved culture media can greatly enhance plating efficiency (LOGEMANN and BERGMANN, 1974; KAO and MICHAYLUK, 1975). We fully expect that plant culture techniques will be developed to increase the plating efficiency of plant cells in the same way that animal cell plating efficiency has been improved over the past 15–20 years.

In mutation studies, physical agents such as ionizing and UV radiation offer some advantage in that dosage can be precisely determined and quantitatively delivered, whereas chemical mutagens must be taken up by the cells and subsequently washed out, making dosimetry more difficult to control. In addition, many chemical mutagens are spontaneously or metabolically degraded in the cellular environment, or must be metabolically activated in order to produce genetic effects, further complicating the dosimetry.

2.1 Ionizing Radiation

There are many different types of X-ray machines ranging in size from the smaller dental units to more powerful therapeutic and research units. The availability of these units accounts for their widespread use in radiation studies. X-rays are

generated by the interaction of an electron beam with a tungsten or molybdenum target. A number of factors (i.e. operating voltage, nature of the target, filtration, tube current, distance to sample) determine the X-ray beam energy spectrum and the dose rate. In general, operating voltages around 100 kV are sufficient for irradiation of cells dispersed in a petri dish. At lower operating voltages, the penetrating power of the beam is correspondingly reduced, so that care must be taken in selection of the appropriate voltage, beam filtration, and sample geometry. The dose rate at the sample position must be measured. This is accomplished with a calibrated ionization meter, or less conveniently by chemical dosimetry. [Refer to ARENA (1971) for an introduction to these considerations.] Gamma rays for biological experiments are generally obtained from isotope sources (e.g. ^{60}Co or ^{137}Cs) which are often housed *within* shielded units; and samples are introduced via a timer-operated elevator. The radiation is more homogeneous and of higher energy than an X-ray beam. Consequently, there is no need to filter out less penetrating "soft" radiation. The dose rate is dependent upon the source-sample geometry and the mass of the isotope. A resulting disadvantage of γ-ray sources is that experiments evaluating dose-rate effects are often not possible since many instruments do not permit changes in the source-to-sample distance. Shielding between the source and sample has been employed to attenuate the γ-rays for dose rate studies (e.g. SPARROW, 1966). Since the dose rate changes with time according to the isotopic decay function (the half-life for ^{60}Co is 5.27 years and for ^{137}Cs, 30 years), this rate must be measured or recalculated frequently. SPARROW (1961) has described the various types, characteristics, and sources of ionizing radiation, including neutrons, β particles, α particles, and protons. The text by ELKIND and WHITMORE (1967) provides a discussion of practical and theoretical aspects of radio-biological experimentation with cultured cells.

The high energy of X and γ-rays reduces problems of differential absorption and penetration of the radiation in the sample. Cells or protoplasts can be conveniently irradiated in a small, sterile, disposable plastic centrifuge tube, thus simplifying subsequent washing of the irradiated material with fresh medium. (Radiation effects on plant growth media are discussed in Sect. 3.5). For irradiation in the absence of oxygen (see Sect. 3.2) the sample can be equilibrated with a sterilized stream of nitrogen gas. Since temperature can seriously alter the biological functions related to radiation effects (see Sect. 3.1), it is often advisable to eliminate variation in temperature, for example, by cooling the sample on ice during irradiation.

2.2 Ultraviolet Radiation

The brief text by JAGGER (1967) provides an excellent introduction to the equipment, methods, and concepts in UV photobiology; workers planning to do UV studies with plant cells will profit from the suggestions and cautions offered therein. Note especially the potential hazard of eye exposure to UV radiation. The most commonly used source of far-UV radiation is the germicidal lamp. These lamps are very effective in inducing mutation or cell killing because the emitted energy (primarily 254-nm radiation) is strongly absorbed by DNA. A

desk lamp fitted with two 15 W lamps (e.g. General Electric Germicidal Lamp) provides an inexpensive and convenient source of far-UV radiation.

For uniform UV exposure, single cells or small aggregates of 2 or 3 cells are absolutely essential and should be dispersed in a thin layer of medium in a petri dish or other suitable container. Constant agitation will further improve the uniformity and reproducibility of irradiation. Of course, the petri dish cover and any other UV-screening material must be removed during irradiation. The high absorbance, scattering, and reflectance of various components (e.g. DNA, RNA, proteins, flavonoids, cuticle) make multicellular organs or large cell aggregates unsuitable for most UV studies. Isolated protoplasts are most nearly ideal; but even so, the concentration and relative localization of organelles and other UV-absorbing components can significantly reduce the actual dose of UV to the nuclear DNA. The UV transmittance of the sample (medium plus cells) should be determined spectrophotometrically and the measured fluences multiplied by a correction factor (MOROWITZ, 1950) to give the incident dose (i.e. fluence) at the level of the cells. When possible, the cells should be irradiated through a UV-transparent medium, such as distilled water or a dilute salt solution, of the minimum thickness to cover the cells. (Of course, this is not possible with protoplasts, which must be maintained in media of high osmotic strength.) Alternatively, cells settled at the bottom of a UV-transmitting culture dish can be irradiated from below (e.g. ERIKSSON, 1967a). These considerations are necessary for accurate dose determinations and will permit more reasonable comparisons to be made on the sensitivities of cells irradiated under conditions employed in different laboratories. Refer to JAGGER (1967) for a further discussion of dosimetry and dose measurement.

Sunlight can be used directly to study effects on cultured cells [e.g. yeast (RESNICK, 1970) and human cells (TROSKO *et al.*, 1970)]. The fluence can be determined with a "sunburn meter" (BILLEN and GREEN, 1975) or with a biological assay utilizing UV-sensitive bacteria (HARM, 1969; BILLEN and FLETCHER, 1974).

3. Factors Modifying the Recovery of Irradiated Cells

Factors which modify postirradiation recovery include those which suggest that recovery requires metabolic activity (e.g. oxygen and temperature), those which suggest a role (or lack of role) for a specific metabolic pathway such as protein synthesis, those which may modify the repair of DNA damage (e.g. certain chemical inhibitors), and those which involve physical modification of irradiations (e.g. dose rate and dose fractionation). In addition, radiolysis products produced in the suspending medium can exert effects on the subsequent growth of both irradiated and unirradiated cells.

3.1 Temperature

Low-temperature incubation following irradiation suppresses repair of chromosomal damage in root tip cells (see WOLFF, 1961) and reduces DNA strand-break

rejoining in murine lymphoma cells (ORMEROD and STEVENS, 1971). The latter authors report that increasing the temperature from 37° C (standard for these cells) to 43° C did not affect the initial rate of repair, but did lead to DNA degradation. In addition, recent work on survival modification of X-irradiated Chinese hamster fibroblast cells at increased temperatures indicates a definite temperature optimum for recovery from sublethal doses in these cells (BEN-HUR *et al.*, 1972).

3.2 Oxygen

Cells irradiated with ionizing radiation in the absence of oxygen generally exhibit a 2/3 reduction in damage or lethality. This "oxygen effect" may be due to the formation of peroxides and their subsequent secondary damaging interactions with cellular constituents (BLOK and LOMAN, 1973). Oxygen can be eliminated by equilibrating the cells with a stream of nitrogen gas before and during irradiation, thus allowing evaluation of the "oxygen effect" on radiation-induced lethality or on some of the molecular aspects of radiation damage (e.g. HOWLAND *et al.*, 1975; Sect. 4.1.1).

3.3 Factors Showing Significance of Specific Metabolic Pathways

The requirement for oxygen during recovery and the enhancement of survival, as measured by the ability of cells to proliferate, with increasing time of split-dose X irradiation in *Oedogonium* (HOWARD, 1968) led to the suggestion that cellular energy metabolism is required for recovery. Experiments with yeast (KIEFER, 1971) offer the strongest evidence that metabolism is the critical requirement for split-dose recovery as measured by colony-forming ability: glucose, which the cells metabolize either aerobically or anaerobically, supports repair at similar rates whether the cells are in an oxygen or nitrogen environment. ORMEROD and STEVENS (1971) found that inhibition of DNA synthesis, RNA synthesis, or protein synthesis was ineffective in reducing DNA strand-break repair in X-irradiated murine lymphoma cells, while inhibition of oxidative phosphorylation reduced single-strand-break repair after high X-ray doses.

TROSKO and HART (1976) have reviewed some effects which inhibit the normal repair of induced DNA damage (see Sect. 4.1) in animal cells. Survival can be reduced and/or mutation frequency increased. Among the chemicals effective in this regard are caffeine, which inhibits postreplication repair and lowers the mutation frequency, and phorbol myristate acetate (a component of croton oil) which inhibits excision-repair, decreases survival, and increases mutation frequency.

3.4 Physical Factors Modifying Recovery

The *rate* at which a dose is delivered to cells can modify the effect of the radiation when a recovery mechanism is operating. The distinction can be made between *acute* and *chronic* doses on the basis of the cell's opportunity to metabolize the

radiation damage being induced. SPIEGEL-ROY and KOCHBA (1973) have observed a dose-rate effect on growth and embryo differentiation in γ-irradiated *Citrus* callus. At rates of 3.1 or 50 Krad/h embryo development was stimulated by a 16-Krad dose, while at 100 Krad/h both growth and embryo differentiation were inhibited. It is possible by cooling the cells on ice to reduce any recovery activity further during an acute irradiation, even at relatively low dose rates.

Dose fractionation is a special case of chronic irradiation in that an interval for recovery intervenes between two or more acute exposures. Dose fractionation can be used to assess the capacity for recovery from radiation damage. If no recovery occurs during the interval between the dose fractions, the cumulative dose will have an effect equivalent to that of a single acute dose of the same total magnitude.

High-LET (linear energy transfer) radiations (e.g. protons and neutrons) are presumed to cause so much damage to any molecule with which they interact that repair seems unlikely. Furthermore, survival curves from such radiations usually are exponential, supporting the observed lack of sparing action by dose splitting (BARENDSON, 1962).

Many other examples of radiosensitivity modification in plants by physical, chemical and biological factors have been cited by SPARROW (1961).

3.5 Media Effects

Radiation can produce chemical changes in culture media in addition to the direct effects produced in the irradiated cells. These "indirect" effects on the growth and differentiation of cultured plant cells have usually been observed only after massive (2000–5000 Krad) doses of ionizing radiation to the sugar component of the media (HOLSTEN *et al.*, 1965; SCHAEVERBEKE *et al.*, 1968; AMMIRATO and STEWARD, 1969; BAJAJ, 1971).

Indirected effects of lower radiation doses have also been reported. VERMA and VAN HUYSTEE (1971a) observed increased growth of irradiated peanut cells when the culture medium was replaced with fresh medium after γ-ray doses of 5–1000 Krad. However, GALUN and RAVEH (1975) observed no effect of γ-irradiated medium on the plating efficiency of irradiated (up to 1.5 Krad) tobacco protoplasts.

Some enhanced morphogenetic effects have also been ascribed to radiolysis products induced in culture media. Embryo formation in *Citrus* ovular callus was equally stimulated by 16 Krad γ-rays to the cells plus medium or to the medium alone, while irradiation of the callus alone was ineffective (SPIEGEL-ROY and KOCHBA, 1973). DEGANI and PICKHOLZ (1973) found that medium irradiated with as low as 0.5 Krad γ-rays permitted shoot development in unirradiated, dark-grown tobacco callus.

ZEEVAART and LEE (1968) reported that *Haplopappus* callus tissue failed to grow on medium which had been exposed to about 10^6 J/m^2 of near-UV from fluorescent "black light" lamps, but these workers did not identify the component(s) of

the medium responsible for the inhibitory effect. WANG and his associates (1975 and references cited therein) have demonstrated that mammalian cells are killed (ca. 1% survival) by toxic photoproducts produced in the culture medium as a result of moderate levels of near-UV irradiation (2×10^4 J/m^2 "black light" or 4×10^4 J/m^2 "daylight" fluorescent lamps). These toxic products appear to result from a riboflavin-sensitized photooxidation of tryptophan or tyrosine. Some complex plant-cell culture media contain these amino acids, but riboflavin is generally not included. KLEIN (1963) observed no effect of UV-irradiated medium on the growth of *Ginkgo* cells.

4. Evaluating the Effects of Radiation on Cultured Cells

Many end points are available for assessing the cellular effect of a particular dose of radiation. These include evaluations at the molecular level (e.g. damage to enzymes or nucleic acids), at the chromosome level (aberrations), at the physiological level (respiration, macromolecular synthesis, hormone synthesis, ion regulation), and at the cellular level (cell growth, cell division, differentiation). Since each involves measurement of a different end point, a direct comparison among the results from these analyses is not possible. Although it appears that some radiation effects may be transient physiological perturbations, the most profound biological effects appear to result from damage to the cell's genetic material—DNA.

The nucleus (specifically the DNA) is the primary radiation-sensitive site in the cell. This conclusion is supported by a large body of experimental evidence including the following:

a) Chromosome aberrations are efficiently induced by ionizing and UV irradiations (see WOLFF, 1961; SPARROW, 1961).

b) Irradiation of the cytoplasm is far less effective in cell killing than irradiation of the nucleus (see ARENA, 1971, pp. 336–337).

c) Radiation that is attenuated by passage through the cytoplasm before reaching the nucleus is less efficient in cell killing (ZIRKLE, 1932).

d) DNA absorbs UV very strongly, and its absorbance spectrum reflects the action spectra for cell killing and mutation (EMMONS and HOLLAENDER, 1939).

e) The large size of the native DNA molecule renders it most susceptible to damage arising from ionizing irradiation (SETLOW and SETLOW, 1972).

f) In bacteria, radiation damage to purified DNA is expressed in DNA transformation experiments (see SETLOW, J. K., 1967).

g) Organisms which are deficient in repair of DNA damage are much more susceptible to radiation-induced killing (see SETLOW and SETLOW, 1972).

Having acknowledged that the DNA is the primary radiosensitive site in the cell, we must also recognize that other effects can be observed as expressions of damaged DNA (e.g. induced mutations) or as expressions of direct damage to secondary sites in the cell (e.g. RNA damage, enzyme inactivation).

4.1 Radiation-induced DNA Damage and Repair

Both ionizing and UV irradiations result in certain more or less well-defined damage in DNA. Ionizing radiation causes single-strand breaks, base damage, double-strand breaks, and, to a lesser extent, interstrand cross-links (see KANAZIR, 1969). UV damage to DNA is primarily in the form of intrastrand dimerization of adjacent pyrimidines, the lesion shown to be important in cell killing (see Setlow, R. B., 1968). Other DNA lesions produced by UV are hydrates of cytosine, cross-links between DNA strands, chain breaks, and DNA-protein cross-links (see SETLOW, R. B., 1972).

It is convenient to discuss repair of ionizing radiation damage and UV damage separately since the respective DNA repair systems appear to be different in certain aspects. A number of comprehensive reviews on DNA damage and repair in microbes and animal cells have been published (e.g. SETLOW, J. K., 1967; HOWARD-FLANDERS, 1968; SETLOW and SETLOW, 1972; CLEAVER, 1974; HART and TROSKO, 1976).

4.1.1 Repair of Ionizing-radiation-induced DNA Damage

MCGRATH and WILLIAMS (1966), examining DNA damage and repair in bacteria, developed a technique which eliminates the need to extract and purify the cellular DNA, thus avoiding mechanical shearing of the DNA. This technique has since been adapted for use with mammalian cells (see SETLOW and SETLOW, 1972; ORMEROD, 1973), but its application to the analysis of DNA in intact plant cells is precluded by the presence of a cellulose cell wall. However, we have applied this method to higher plant cells by utilizing isolated protoplasts of cultured wild carrot cells (HOWLAND *et al.*, 1975). Cells containing radioisotope-labeled DNA are enzymatically converted to protoplasts, irradiated, and placed directly on the surface of an alkaline sucrose gradient. The protoplasts lyse, the DNA is dissociated from the chromosomal proteins and RNA, and the DNA is denatured by the alkaline conditions. The gradient is then centrifuged, and the DNA sedimentation velocity is determined by the distribution of radioactive DNA in the gradient. The quantitative determination of induced strand breaks is based on the reduction in single-strand molecular weight with increasing dose of ionizing radiation. Likewise, repair of strand breaks is seen as the recovery of higher-molecular-weight DNA with postirradiation incubation of the cells prior to lysis on the gradient. For γ irradiation of wild carrot protoplasts, the induction of single-strand breaks is linear with increasing doses and amounts to 1.2×10^{-12} breaks/dalton DNA/rad. In the absence of oxygen, the yield of strand breaks is reduced by 2/3 to 0.4×10^{-12}/dalton DNA/rad (oxygen effect, see Sect. 3.2). It should be noted that single-strand breaks detected on alkaline sucrose gradients after exposure of cells to ionizing radiation are of two types: actual interruptions in the polynucleotide chains, and alkali-labile sites such as those which result from base loss (see TOWN *et al.*, 1973). If the irradiated (20 Krad) wild carrot protoplasts are allowed to incubate at 28° C, the single-strand breaks are rapidly repaired (Figs. 1 and 2), so that by 60 min none of the original lesions are detected. All organisms which have been examined thus far, including a higher plant, normally have the

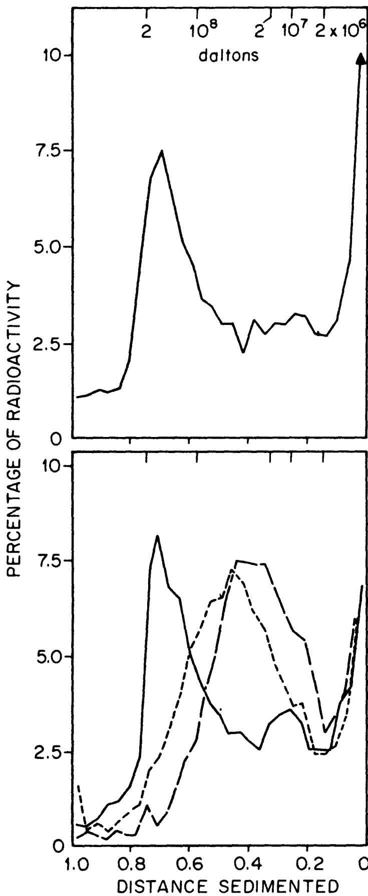

Fig. 1. Sedimentation profiles in alkaline sucrose gradients of wild carrot protoplast DNA before irradiation (*upper panel*); immediately after 20 Krad γ-rays at 0° C (— — —); and after post-irradiation incubation at 28° C for 5 min (------) or 47 min (———) (*lower panel*). (From HOWLAND *et al.*, 1975, with permission from Elsevier Publ. Co.)

capacity to repair strand breaks in DNA (see SETLOW and SETLOW, 1972; HOWLAND *et al.*, 1975). However, recent data on nucleated nondividing chick erythrocytes indicate that these cells have a decreased ability to rejoin DNA strand breaks and appear to accumulate breaks in their DNA with age (KARRAN and ORMEROD, 1973).

Only one higher plant system other than wild carrot has been examined—*Vicia faba* root tips. In that system there is an apparent absence of ionizing-radiation-induced DNA repair synthesis (WOLFF and SCOTT, 1969; PAINTER and WOLFF, 1973). Although these negative results may result from technical difficulties in conducting the experiments (see HOWLAND *et al.*, 1975; HOWLAND and YETTE, 1975), it may be that wild carrot and *Vicia* represent extremes in the range of ionizing-radiation-damage repair capacities to be found in plants.

Since most cells efficiently repair single-strand breaks in DNA, ORMEROD and STEVENS (1971) suggest that lesions other than single-strand breaks (e.g. double-strand breaks, base damage) are responsible for radiation-induced cell killing (see also HART and TROSKO, 1976). Another major class of DNA damage induced by ionizing radiation is "base damage". CERRUTTI and his associates have character-

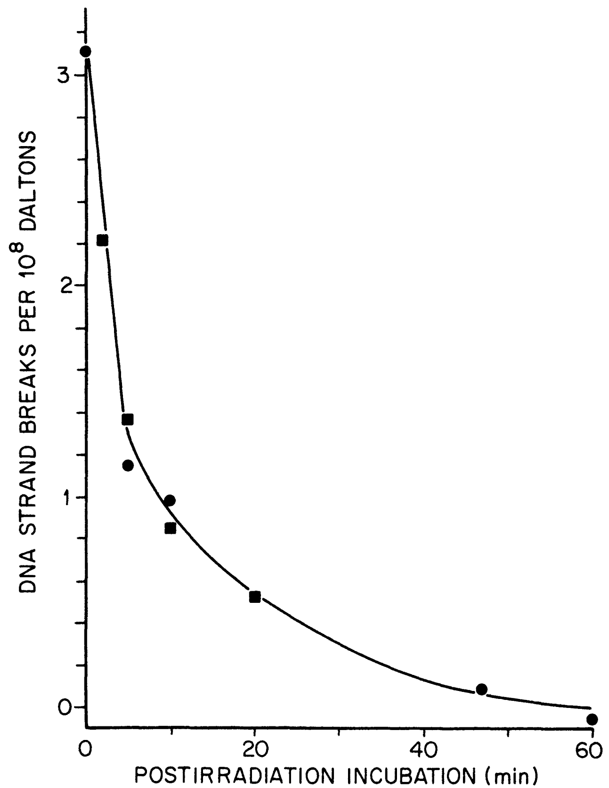

Fig. 2. Kinetics of repair at 28° C of strand breaks (plus alkali-labile bonds) in DNA of wild carrot protoplasts after 20 Krad of γ irradiation at 0° C. The data from two separate experiments are plotted (● and ■). (From HOWLAND *et al.*, 1975, with permission from Elsevier Publ. Co.)

ized some specific types of base damage and have developed methods to assay for their excision from the DNA of mammalian cells (see MATTERN *et al.*, 1975). These assays should be directly applicable to the characterization of ionizing radiation damage and repair in plant cell DNA. WILKINS (1973) and WILKINS and HART (1974) have described an assay for the repair of lesions in DNA utilizing a DNA endonuclease that acts on ionizing-radiation-induced lesions in DNA – so-called endonuclease-sensitive sites. These sites can be detected as strand breaks after nuclease treatment when the DNA is sedimented in a gradient of alkaline sucrose. A reduction in the number of endonuclease-sensitive sites with time is indicative of repair of these lesions.

4.1.2 Repair of UV-induced DNA Damage

The biologically most important UV-induced lesion in DNA is the "pyrimidine dimer" (see Sect. 4.1). Pyrimidine dimers in DNA are induced linearly with increasing UV fluence until very high doses are reached. In isolated protoplasts of

wild carrot cells this amounts to about 2×10^4 dimers/cell per J/m^2 UV dose (HOWLAND, 1975). The number of dimers per cell resulting from a specific UV exposure can be larger or smaller depending on the cellular DNA content and base composition and the degree of attenuation imposed by the optical properties of the irradiated cells and by the irradiation conditions.

Cells can recover from pyrimidine dimer damage in at least three different ways: (1) photoreactivation; (2) excision repair; and (3) postreplication repair.

4.1.2.1 Photoreactivation of Pyrimidine Dimers

Pyrimidine dimers in DNA can be monomerized in situ by the action of photoreactivating enzyme and visible light. The nature of this repair activity and its phylogenetic distribution have been reviewed (COOK, 1970; RUPERT, 1975). Photoreactivation (PR) of pyrimidine dimers has been demonstrated in cultured cells of *Nicotiana tabacum* (TROSKO and MANSOUR, 1968), *Ginkgo biloba* (TROSKO and MANSOUR, 1969a), and *Daucus carota* (wild carrot) (HOWLAND, 1975), but not in *Haplopappus gracilis* cells (TROSKO and MANSOUR, 1968). However, the negative results obtained with *Haplopappus* may be a function of the very high UV doses employed (see Sect. 4.6.3).

The action spectrum for PR of UV-induced lethality in cultured *Ginkgo* cells (KLEIN, 1963; Sect. 4.6.1) shows maximum efficiency at about 420 nm, similar to PR in a fungus [*Streptomyces griseus* (KELNER, 1949; JAGGER *et al.*, 1970)], and in a blue-green alga [*Agmenellum quadruplicatum* (VAN BAALEN, 1968)]. In contrast, PR in *Escherichia coli* is maximum in the region of 380 nm (see JAGGER, 1967; RUPERT, 1975). This difference should be taken into account when PR is attempted with higher plant cells, since a common source which has been used for PR studies is the fluorescent "black light" (e.g. General Electric BLB, λ = ca. 300–400 nm, $\lambda_{max} = 360$ nm). PR of UV-induced pyrimidine dimers in the DNA of wild carrot protoplasts is illustrated in Figure 3. PR enzyme in wild carrot cells can utilize "black light" even though the optimally effective spectral region may be at longer than the maximum wavelength for these lamps. Complete repair (i.e. excision plus PR) of a moderate level of UV damage has been shown for wild carrot protoplasts exposed to cool-white fluorescent light for 24 h, whereas after this UV dose, dark-repair (Sect. 4.1.2.2) alone gave only about 60% dimer removal from the DNA (HOWLAND, 1975).

Monomerization of dimers in DNA constitutes a demonstration of "direct" photoenzymatic repair. "Indirect PR" has been observed for many other biological effects after UV irradiation, but can be distinguished on the basis of several criteria (SETLOW, R. B., 1968; COOK, 1970). Most clearly, in order to be considered "direct", the PR light is effective only when administered *after* the damaging UV dose. PR which is effective prior to the UV irradiation is necessarily acting indirectly, and is termed photoprotection (see SETLOW, J. K., 1967; JAGGER, 1967; COOK, 1970). Photoprotection by blue and red light has been reported for growth of UV-irradiated *Ginkgo* tissue (KLEIN, 1963). As a result of photoprotection it is possible that cells propagated in the dark will display a higher UV sensitivity than those which have been grown with illumination.

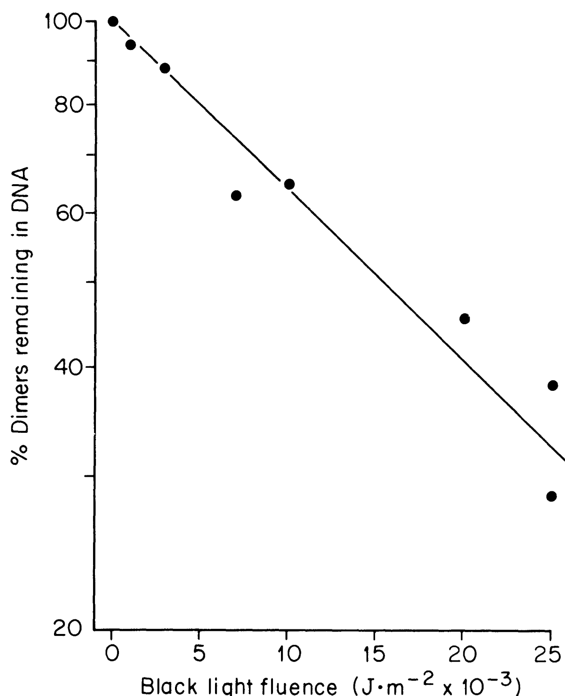

Fig. 3. Photoreactivation of UV-induced (fluence = 70 J/m^2) pyrimidine dimers in the DNA of wild carrot protoplasts. Black light (λ = 300–400 nm) was obtained from General Electric BLB lamps at a fluence rate of 126 J/m^2/min after passing through 1-cm plate glass. Conditions for dimer analysis were as described previously. (HOWLAND, 1975; unpublished)

Since PR is apparently specific for the monomerization of pyrimidine dimers in polynucleotides (COOK, 1970), direct PR can be employed as a diagnostic tool for evaluating the possible role of pyrimidine dimers in producing various UV-induced effects. If direct PR reduces or eliminates the UV effect, then the DNA is implicated as the target molecule and the pyrimidine dimer as the important lesion in producing the effect (e.g. HART and SETLOW, R. B., 1974b).

4.1.2.2 Excision-Repair of Pyrimidine Dimers

The second major mechanism for repair of dimer damage in DNA is excision-repair. The pathway for this repair activity has been studied in most detail in bacteria, but the essential features appear to apply to eukaryotic cells as well. The first step involves an endonuclease which incises the DNA strand near the lesion. Then exonucleolytic action removes the dimer as well as a number of adjacent nucleotides. Polymerase resynthesizes the DNA which has been excised, using the opposite strand as a template. Finally, DNA ligase seals the remaining nick. (For reviews of this process, see GROSSMAN, 1974; HART and TROSKO, 1976.) Assays for this repair activity include (1) chromatographic analysis of dimers in DNA (CARRIER and SETLOW, 1971), (2) autoradiographic detection of unscheduled (i.e. re-

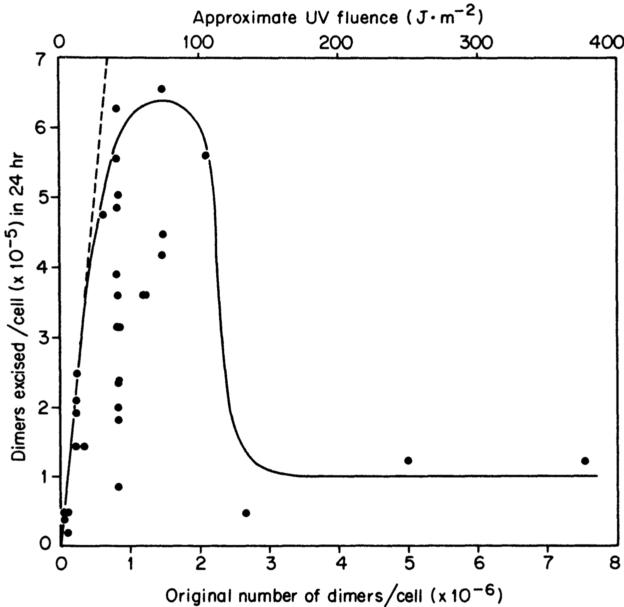

Fig. 4. Number of UV-induced pyrimidine dimers in wild carrot protoplast DNA excised during 24-h postirradiation incubation (28° C, dark). Values for individual analyses (●) were determined as before (HOWLAND, 1975). Complete dimer excision (*broken line*) and maximum dimer excision observed (*solid line*) are indicated. (HOWLAND, unpublished)

pair) DNA synthesis (see CLEAVER, 1974), (3) measurement of DNA repair replication by [^{3}H]bromodeoxyuridine (BrdUrd) incorporation and subsequent equilibrium centrifugation of the DNA (see CLEAVER, 1974), and (4) incorporation of BrdUrd during repair replication followed by photolysis of the repaired (i.e. BrdUrd-substituted) regions with 313-nm radiation and analysis of the resulting reduction in DNA single-strand molecular weight on alakaline sucrose gradients (REGAN *et al.*, 1971).

Like all known chemically induced DNA damage, some types of UV-induced DNA damage are not photoreactivable (e.g. SETLOW, J. K., 1967; RESNICK, 1970). Consequently, excision-repair competence becomes important to the cell in dealing with a variety of DNA lesions, including dimers. Although several earlier attempts to find dimer excision in cultured plant cells gave negative results (TROSKO and MANSOUR, 1968, 1969a), this capacity recently has been demonstrated in isolated protoplasts of cultured wild carrot cells (HOWLAND, 1975). Figure 4 illustrates that after low UV doses, dimers are efficiently excised (~100%), but that after higher doses, excision is drastically reduced.

Since the excision-repair process removes intact pyrimidine dimers from DNA, the appearance of dimers in the acid-soluble cell fraction confirms that repair is occurring via excision and not by PR (Fig. 5). These data also indicate that in cultured wild carrot protoplasts the rate of dimer excision is initially very rapid, but is essentially zero after 24 h.

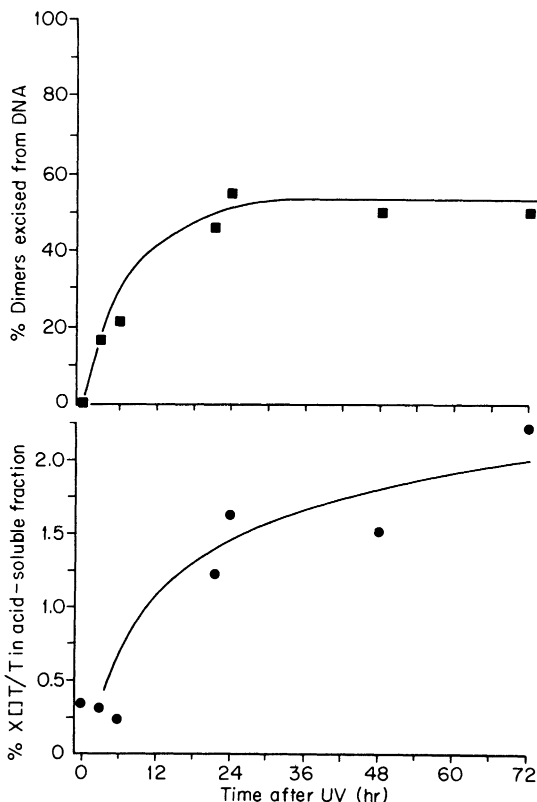

Fig. 5. Kinetics of pyrimidine dimer excision from the DNA of wild carrot protoplasts (upper panel) and appearance of excised dimers in the cold-acid-soluble cell fraction (lower panel). The UV fluence applied at time = 0 was 42 J/m^2. Conditions for dimer analysis were as reported previously (HOWLAND, 1975).

$$\% \text{X[]T/T} = \frac{\text{radioactivity in thymine-containing dimers}}{\text{radioactivity in thymine}} \times 100 .$$

(HOWLAND, unpublished)

Among the placental mammals a wide range in excision-repair capacity has been found, with mouse cells having the least active and human cells exhibiting the most active excision-repair (HART and SETLOW, 1974a). Work has just begun to determine if a similar variation exists among the higher plants. Excision-repair has also been found in *Haplopappus gracilis* and *Nicotiana tabacum* cells and in *Petunia hybrida* protoplasts (HOWLAND, unpublished).

Mutant cells unable to excise pyrimidine dimers display increased sensitivity to UV. Such mutants are known in bacteria and yeast and also in human cells [i.e. xeroderma pigmentosum (CLEAVER, 1968)]; but as yet none has been observed in higher plants. Haploid cell lines derived from anther culture (see Chap. II.1 of this Vol.) provide the material to select induced mutations in this important function. These mutants would be valuable in advancing our understanding of excision-repair in higher eukaryotes, as well as providing a tool for examining other pathways for DNA repair (e.g. postreplication repair).

4.1.2.3 Postreplication Repair

Dimers which are not photoreactivated or repaired by excision constitute blocks to normal semiconservative DNA synthesis. In mammalian cells this has been shown to result in the synthesis of smaller-than-normal DNA molecules. At later times the block is by-passed and the DNA is joined to form the normal high-molecular-weight DNA (see LEHMANN, 1974). The details of postreplication repair in mammalian cells are currently under investigation in several laboratories, and some of the concepts already put forth have been questioned (PAINTER, 1974). Evidence of postreplication repair activity has recently been observed in protoplasts of cultured wild carrot cells (HOWLAND, unpublished).

4.2 Mitotic Delay and Reduced Rate of DNA Synthesis

UV-induced dimers inhibit normal DNA synthesis in many cells. TROSKO and MANSOUR (1969b), following the rate of [^{3}H]thymidine incorporation into cultured tobacco cells, found that DNA synthesis is reduced after irradiation, but that PR could (partially) reverse this inhibition. These data implicate pyrimidine dimers as being the important lesion in UV inhibition of DNA synthesis, and are consistent with more extensive data from bacterial and mammalian systems (see KANAZIR, 1969). OHYAMA *et al.* (1974) have observed UV inhibition of DNA synthesis which displays kinetics similar to the UV inactivation of colony-forming ability in isolated soybean protoplasts. In cells that are not undergoing normal semiconservative DNA synthesis and are able to excise pyrimidine dimers from their DNA, there is a UV-stimulated increase in [^{3}H]thymidine incorporation (repair replication, Sect. 4.1.2.2). At low to moderate UV doses, the UV-stimulated repair synthesis may obscure any UV inhibition of normal DNA synthesis. This is true in cultured wild carrot protoplasts where normal DNA replication is much reduced during the first day after enzymatic isolation (HOWLAND, unpublished).

ERIKSSON (1967b) observed a reduction in mitotic index in *Haplopappus* cells following X-ray or UV exposure. Division delay per se has not been described in samples of irradiated plant cells in vitro, but observations on cultured mammalian cells (see PAINTER, 1973) and on algae (HART, 1971b) indicate that some cells can recover from radiation damage after exhibiting a delay in cell division. In these cases the delay period (in cells that can recover) amounts to up to one cell cycle length (see PAINTER, 1973). ERIKSSON'S (1967a) results with survival of irradiated *Haplopappus* cells suggest that the radiation-induced mitotic inhibition is overcome since many cells can recover to form colonies.

4.3 Radiation-induced Chromosome Aberrations

SUNDERLAND (1973) has discussed the high degree of chromosomal variation usually observed in cultured plant cells. A high frequency of "spontaneous" chromosome aberrations makes cultured plant cells less valuable as potential cytoge-

netic material in which one could evaluate the induced rate of chromosomal aberrations following experimental treatment (e.g. WOLFF, 1961). This spontaneous aberration level can be affected by the culture conditions (STREET, 1973, p.429). SINGH and HARVEY (1975) have reported strong selection against polyploid and aneuploid *Haplopappus* cells under conditions which favor rapid, homogeneous growth (i.e. suspension culture, frequent transfers). It is possible that under the appropriate culture conditions, cultured cells of many other plant species could be maintained with a stable diploid or haploid karyotype.

ERIKSSON (1967a) evaluated the effects of X-rays and UV on production of chromosome aberrations in *Haplopappus* cells and demonstrated the potential application of cultured plant cells to cytogenetic analyses (see also ERIKSSON, 1967b).

4.4 Induction of Giant Cells by Irradiation

Cells irradiated with doses high enough to block cell division often retain the capacity for continued growth. After massive γ irradiation of dry wheat seeds, the embryo can germinate without DNA synthesis or cell division and grow by exaggerated cell enlargement to form a seedling that is surprisingly normal morphologically, biochemically, and physiologically (HABER, 1972). Giant cell formation has been observed in cultured bacterial, animal, and plant cells (VERMA and VAN HUYSTEE, 1971a, and references cited therein). Massive γ irradiation (550 Krad) of cultured peanut cells increased the frequency of giant cells from 10 to 60% of the population (VERMA and VAN HUYSTEE, 1971a); these giant cells had grown to 10–15 times their normal size. Giant cell formation has also been observed in γ-irradiated *Phaseolus vulgaris* culture (BAJAJ, 1973) and in X-irradiated Jerusalem artichoke tissue (JONARD, 1959). Even though no such radiation-induced increases in cell size of microcultured tobacco cells (CRUZ and HILDEBRANDT, 1968) or grape stem callus (ARYA and HILDEBRANDT, 1969) have been observed, giant cell formation may still, at least in part, account for the observed increases in fresh or dry weight of cultures exposed to high doses of ionizing radiation (see also Sect. 4.6.1).

4.5 Other Physiological Effects of Radiation

In addition to the previously discussed inhibition of normal DNA synthesis, radiation can also depress the rates of RNA and protein synthesis. UV irradiation of soybean protoplasts results in dramatic reductions in the incorporation (into acid-insoluble material) of [^{14}C]uridine and L-[^{14}C]alanine (OHYAMA *et al.*, 1974). These effects were observed in the range of UV fluences which also inhibit colony-forming ability.

In *Nicotiana* suspension cultures, D_{37} (i.e. dose required to reduce the measured parameter to 37% of the control level) for inhibition of protein synthesis is reached at an incident fluence of 388 J/m^2 UV (MURPHY *et al.*, 1975). The samples used in these experiments were present as aggregates of 1–35 cells, with about

one-half larger than five cells in diameter. The rapid UV inhibition of amino acid incorporation does not appear to result from inhibition of respiration, reduction in endogenous ATP levels, reduced uptake of labeled amino acids, or inhibition of messenger RNA synthesis. Direct UV damage to polysomes, as assayed in a cell-free system, accounts for less than one-half of the inhibition observed with intact cells (MURPHY *et al.*, 1975), suggesting that some other, as yet unidentified mechanism(s) is responsible. Additionally, it is likely that inhibition of amino acid incorporation occurs primarily in those cells which are unshielded from the UV radiation (i.e. free cells and cells at the exterior of aggregates). Autoradiographic analysis could be used to investigate this latter possibility.

VERMA and VAN HUYSTEE (1971b) observed that the initial 50% depression of protein synthesis in massively irradiated (500 Krad) peanut cells disappeared after one week of postirradiation incubation. By two weeks protein synthesis exhibited a transient increase to ca. 150% of control level, falling to the control level at 3 weeks. However, chromatographic and electrophoretic analyses indicated that the distribution of proteins was clearly abnormal in the cells which had been irradiated. These authors termed this "aberrant recovery" of protein synthesis.

Ionizing radiation has also been employed to reduce the endogenous level of indoleacetic acid in plant cells. Crown-gall tumor tissue, which grows on culture media lacking auxin, can be made auxin-dependent by a 1-Krad dose of X-rays (KLEIN and VOGEL, 1956). BAJAJ *et al.* (1970b) reported that the growth of bean callus, as measured by increase in dry weight, was slightly (5%) stimulated by a 0.5 Krad γ-ray dose; but they did not determine whether this effect was due to stimulation of cell division, cell expansion or both. Stimulation of differentiation in cultured plant tissues has been observed after ionizing radiation doses up to 20 Krad (DEGANI and PICKHOLZ, 1973; SPIEGEL-ROY and KOCHBA, 1973; NORREEL and RAO, 1974), but this appears to be an indirect effect of the irradiated medium on the plant cells (see Sect. 3.5).

4.6 Lethality as an End Point for Radiation Damage

The ultimate radiation effect is, of course, cell killing. A lethal event is usually assayed as the loss of cellular reproductive capacity (i.e. colony-forming ability) in microbial and mammalian cell systems. In the absence of a reliable cell-plating assay (see Sect. 2) other, less-satisfactory measures have been employed in evaluating the radiosensitivity of cultured plant cells.

4.6.1 Measurement of Survival by Mass Increase

Many workers have measured increase in fresh and/or dry weight or sedimented cell volume as a parameter of survival. Although it is certainly true that an absence of fresh- or dry-weight increase in an irradiated culture (compared with control cultures) is indicative of (near) zero survival, it is not possible to interpret intermediate growth increases. Among the confounding factors are: division delay (Sect. 4.2); giant cell formation (Sect. 4.4); transient effects on DNA, RNA, and protein synthesis (Sect. 4.5); and possible temporary rescue by cross-feeding

among adjacent cells. Unfortunately, this combination of undefined elements precludes the interpretation of such data in terms of cell survival.

VENKETESWARAN and PARTANEN (1966), examining the growth response of γ-irradiated tobacco suspension cultures, observed a significant depression in growth attained after doses beyond 2 Krad. The apparent D_{37} for growth of this tissue is about 18 Krad. BAJAJ *et al.* (1970b) obtained similar results with *Phaseolus* callus (apparent D_{37} = ca. 19 Krad). A marked contrast exists between these data and those obtained for single-cell survival of cultured mammalian cells, where D_{37} of less than 1 Krad are observed for ionizing radiation (see ELKIND and WHITMORE, 1967; SETLOW and SETLOW, 1972). Attempts to quantitatively interpret radiosensitivity data obtained via weight-increase measurements of cultured plant tissue have led to the (probably erroneous) conclusion that cultured cells are more radioresistant than those in the intact plant (BAJAJ and BOPP, 1971; DULIEU, 1972).

KLEIN (1963) irradiated *Ginkgo* cells with UV and assayed for effects on growth of the cultured tissue by dry-weight increase. The apparent D_{37} in the absence of PR was reached at an incident fluence of ca. 40 J/m^2. TROSKO and MANSOUR (1968) demonstrated a UV-induced growth inhibition, as measured by reduction in fresh weight increase, which could be partially reversed in suspension-cultured tobacco (but not *Haplopappus*) by PR (but see Sect. 4.6.3).

4.6.2 Cellular Parameters Used in Survival Assays

We have acknowledged that quantitative survival assays must reflect the reproductive potential of single cells. Although the most direct and generally accepted method is to measure colony-forming ability of single cells, there are other parameters which can be estimated without requiring strictly single-cell preparation or high plating efficiency; but such assays do not necessarily reflect the reproductive potential of the irradiated cells. In almost every case they tend to overestimate the true cellular survival. These assays include cytoplasmic streaming and vital staining. CRUZ and HILDEBRANDT (1968) observed that the D_{37} for cytoplasmic streaming in microcultured tobacco cells exposed to γ-rays was 250–500 Krad at two weeks postirradiation. Cells infected with tobacco mosaic virus showed an increased sensitivity for this parameter (D_{37} = ca. 100 Krad), but even this is far beyond the radiosensitivity observed in a cell-plating assay (see Sect. 4.6.3). ARYA and HILDEBRANDT (1969) used the cytoplasmic streaming assay to compare the radiosensitivities of cultured normal grape stem cells and leaf gall cells. The gall cells appeared to be somewhat more radiosensitive than the normal cells, although the differential was slight (D_{37}'s = 0.5–1.0 Krad). In this case the radiosensitivities were similar to those found with single-cell-plating assays (see Sect. 4.6.3).

Viability of individual cultured plant cells or protoplasts can be assayed using dye exclusion (GAFF and OKONG'O-OGOLA, 1971; WIDHOLM, 1972b), fluorescence (WIDHOLM, 1972b), or dye reduction (TOWILL and MAZUR, 1975). Dead cells are unable to exclude colloid dyes (e.g. phenosafranine, trypan blue, Evan's blue); they do not enzymatically cleave fluorescein diacetate to fluoroscein (which fluoresces); nor do they reduce 2,3,5-triphenyltetrazolium chloride to a colored product (red formazan).

Vital staining offers the advantages of being rapid and quantitative, but serious questions must be raised about the relationship of these assays to survival as measured by cell proliferation. TOWILL and MAZUR (1975) have found a good correlation between tetrazolium dye reduction and plating efficiency (*Haplopappus* cells) or regrowth (*Acer saccharum* cultures) when frozen samples are compared. However, vital stains (see WIDHOLM, 1972b) do not adequately discriminate between viable and lethally irradiated wild carrot or soybean protoplasts [UV and γ-rays (HOWLAND, unpublished data)]. In agreement with these observations is the comment of OHYAMA *et al.* (1974) that soybean protoplasts irradiated with supralethal UV doses (based on colony-forming ability) retain the ability to exclude trypan blue. Since cell survival is to be based on reproductive capacity, it is not surprising that vital stains fail to distinguish between cells which are merely physiologically active and normal cells which are capable of proliferation [see also the "gamma plantlet" (HABER, 1972)].

4.6.3 Survival Measurement by Single-cell Proliferation

ERIKSSON (1967b) utilized a plating assay to evaluate the lethal effects of X-rays and UV on a suspension culture of *Haplopappus* which had been filtered (60 μm) to remove aggregates of more than four cells. His data indicate apparent D_{37}s of ca. 3 Krad (X rays) and 300 J/m^2 (UV, growth in white light), but ERIKSSON suggests that even these estimates may be somewhat inflated since the plated individuals consisted of mostly 2–4 cell aggregates. In addition, the presence of anthocyanin in the cells would reduce UV sensitivity by screening the radiation reaching the DNA.

Collection of the 20- to 53-μm filter fraction of a highly disaggregated *Haplopappus* suspension culture yielded the following distribution: 70% single cells, 25% doubles, 5% three-celled aggregates, and less than 1% four-celled or larger aggregates. Survival after UV irradiation was determined microscopically as the proportion of plated cells (plus aggregates) that had grown to form colonies of at least 10 cells (Fig. 6). When corrected for the probability of totally inactivating the multicelled aggregates as well as the single cells, these data indicate D_{37}s of approximately 80 and 260 J/m^2 without and with PR, respectively (HOWLAND, unpublished).

Corrections were made by applying the function:

$$I_0 = pf_1 + p^2 f_2 + p^3 f_3$$

where I_0 is the observed fractional inactivation of colony formation at a UV dose; f_1, f_2, and f_3 are the proportions of initially irradiated single cells (0.70), doubles (0.25), and three-celled aggregates (0.05), respectively; and p is the actual probability of inactivation for a single cell at that UV dose. The value of p is assumed to be the same for isolated cells and for each cell in an aggregate.

The development of techniques for protoplast isolation and plating (e.g. NAGATA and TAKEBE, 1971; RAVEH *et al.*, 1973; OHYAMA *et al.*, 1974) has provided a system in which to evaluate the reproductive potential (i.e. colony-forming ability) of truly isolated cells. GALUN and RAVEH (1975; Fig. 7) have determined the dose-survival relationship for X-irradiated tobacco mesophyll protoplasts. D_{37}s of

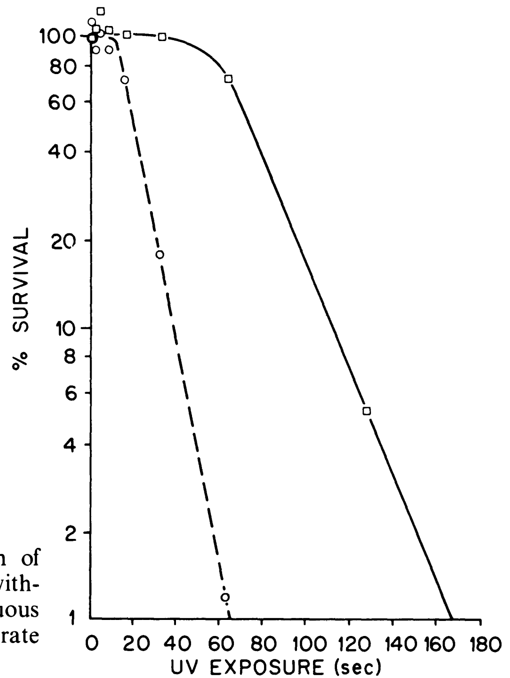

Fig. 6. Dose-survival for colony formation of UV-irradiated *Haplopappus* cells grown without photoreactivation (○) or with continuous white light (□). The incident UV fluence rate was 3.4 $J/m^2/sec$. (HOWLAND, unpublished)

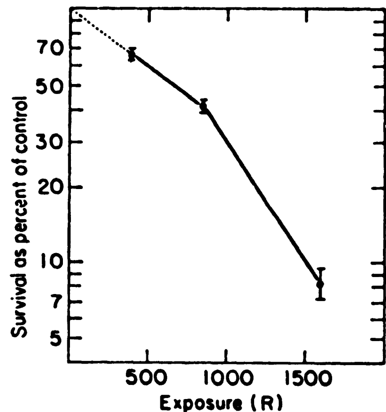

Fig. 7. Survival of diploid tobacco cells exposed to different X-ray doses on the third day after protoplast isolation. Plating density was 3×10^4 protoplasts/ml. (From GALUN and RAVEH, 1975, with permission from the authors and Pergamon Press)

between 0.5 and 1.0 Krad were observed, comparable to the radiosensitivities of cultured mammalian cells (see SETLOW and SETLOW, 1972). Haploid protoplasts were found to be somewhat more radiosensitive than their diploid counterparts, but the major determinant of radiosensitivity in this study was apparently the cell cycle stage at the time of irradiation (GALUN and RAVEH, 1975). Mesophyll cells of mature tobacco leaves do not normally divide, but when protoplasts are isolated and cultured they begin to progress through the cell cycle with some degree of synchrony, commencing a wave of nuclear division on the third day after isolation. GALUN and RAVEH (1975) also cite unpublished data of ZELCER indicat-

ing that DNA synthesis begins in these cultures at about 24 h and reaches a maximum after 40 h. Radiosensitivity of both haploid and diploid cells was highest on the second day after initiation of the cultures; this peak was presumably correlated with the DNA synthetic period in these cells.

In contrast, BURHOLT and VAN'T HOF (1974) found that initiation of proliferation was correlated with a decreased radiosensitivity, while ploidy difference (diploid vs diploid-plus-polyploid) gave no indication of differential radiosensitivity. In this work, pea root segments were cultured on media which selected for proliferation of diploid pericycle cells only (no kinetin) or for proliferation of both the diploid pericycle cells and polyploid cortical cells (kinetin added). The degree of cell proliferation was determined by macerating explants in 20% chromic acid at 37° C for 24 h, resuspending in saline, and passing the sample through a 23-guage needle. The separated single cells were then counted using an electronic particle counter. Although this technique does eliminate some of the possible confusion encountered with measurements of fresh- or dry-weight increase (e.g. giant cell formation) there are still questions as to the possible contributions by (1) a radioresistant subpopulation of cells (e.g. cell-cycle-stage-related radiosensitivity), (2) cells that recover from radiation exposure and initiate proliferation at a later time, and (3) limited proliferation of lethally irradiated cells. BURHOLT and VAN'T HOF (1974) were able to distinguish among these possibilities regarding their observation of a limited but constant percent increase in cell number observed at doses above 3 Krad in all their experiments. Since at the time of sampling (7 days postirradiation) the mitotic index in this tissue was at or near zero for the doses over 3 Krad, it follows that pea cells can temporarily divide after being lethally irradiated, and that the extent of this temporary proliferation is independent of dose over the range tested (3–6 Krad).

Since lethally irradiated cells can undergo at least 1 or 2 cell divisions before dying after ionizing radiation (see PAINTER, 1973), it is necessary to ignore colonies which have not reached a predetermined minimum size. GALUN and RAVEH

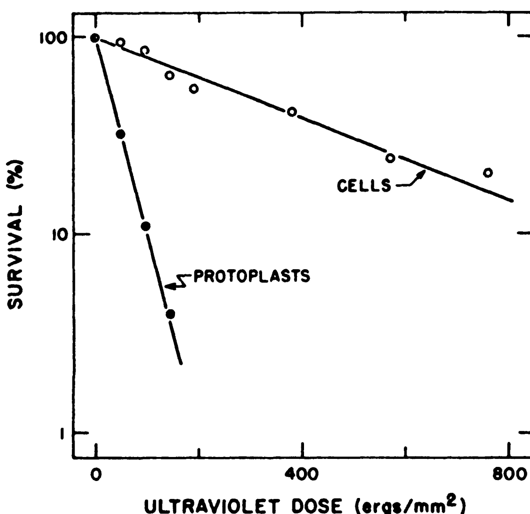

Fig. 8. Survival of soybean protoplasts and cultured cells as measured by colony-forming ability after exposure to increasing UV doses. (From OHYAMA *et al.*, 1974, with permission from the authors and Pergamon Press)

(1975) acknowledged this effect and employed the criterion of ≧ 20 cells/colony for survival. VARDI *et al.* (1975) have also determined the radiosensitivity of cultured citrus protoplasts, observing a D_{37} of ca. 4 Krad for loss of colony-forming ability.

OHYAMA *et al.* (1974; Fig. 8) determined the colony-forming ability of UV-irradiated soybean protoplasts cultured in the dark (i.e. no photoreactivation). An incident fluence of ca. 4.4 J/m^2 yielded a 37% reduction in survival. The UV fluences at the level of the cells were actually even less than those reported, due to UV absorbance and scattering properties of the suspending medium and of cytoplasmic constituents (see Sect. 2.2). Applying this correction indicates a D_{37} of ca. 3 J/m^2, comparable to the UV sensitivity of UV-sensitive mutants in yeast (COX and PARRY, 1968). OHYAMA *et al.* (1974) also observed that colony formation by cell aggregates of this tissue gives an impression of lower UV sensitivity, probably due to shielding effects and the cell multiplicity in the plated clusters. However, neither survival curve displays a shoulder in the low-dose range as is characteristic of DNA repair-competent cells.

4.7 Radiation-induced Mutations in Cultured Plant Cells

Cultured plant cells are increasingly being utilized in genetic studies at both the basic and applied levels (e.g. NICKELL and TORREY, 1969; CARLSON, 1973; SMITH, 1974; BOTTINO, 1975; CARLSON and POLACCO, 1975). First, the plant tissue must sis: the dosimetry can be more easily determined, it is often safer to carry out the mutagenic treatment, and there is no residual mutagen left in the irradiated sample after exposure. Chemical mutagens, on the other hand, offer their own advantages, especially their ability to induce certain relatively specific types of DNA alterations.

Recent reviews have pointed out the potentials and the current limitations on the use of cell cultures in plant improvement research (MELCHERS, 1972; BAJAJ, 1974; BOTTINO, 1975, CARLSON and POLACCO, 1975). First, the plant tissue must be established in culture with a high degree of chromosomal stability. Second, the culture of haploid cells is required in order to select directly for recessive mutations in vitro. Third, single cells (or protoplasts) must be isolated and cultured under conditions which permit the induction of mutations and the application of the appropriate selective screen to identify and isolate the (rare) desired mutant cells as clones. A high degree of genetic resolution can readily be achieved by screening a population of 10^6–10^8 individual plant cells. Finally, to allow parallel formal genetic analysis and to be useful in a plant breeding program, a plant cell culture must give the option of regenerating shoots or plants from the mutant clones selected in vitro. Each of these goals has already been attained in the case of tobacco, but much more work is needed so that this powerful genetic approach may be effectively applied toward the improvement of other important crop plants.

Although chemical mutagenesis has been employed to induce mutant plant cells in culture (see CHALEFF and CARLSON, 1974), relatively little use has been made of radiation mutagenesis. NITSCH and his associates (NITSCH *et al.*, 1969;

NITSCH, 1969, 1972) demonstrated that mutant plants can be obtained by 1.5–3 Krad doses of γ irradiation to haploid microspores or plantlets in cultured tobacco anthers. Mutant characteristics observed included leaf variegation, albinism, and flower color and petal shape variations. DEVREUX and SACCARDO (1971) recovered a high frequency of presumably induced mutations from irradiated (1 Krad X-rays) anther cultures.

J. M. WIDHOLM (pers. comm.) has employed UV to induce mutations to amino-acid-analog resistance in cultured wild carrot cells (see WIDHOLM, 1972a). A mutagenic exposure was selected so as to reduce cell survival by about one-half, as evaluated by vital staining (WIDHOLM, 1972b). This treatment increased the mutation frequency about 10-fold over the spontaneous rate.

ERIKSSON (1967b) reported the recovery, from a UV-irradiated *Haplopappus* culture, of a stable variant exhibiting an altered karyotype and a high propensity for anthocyanin production.

UV mutagenesis, because of its specificity, availability, convenience, and relative safety, should find wide application in studies with isolated plant cells. More is known about the molecular effects of UV on DNA (i.e. pyrimidine dimer induction) and about the repair of these DNA lesions than for any other agent (see Sect. 4.1.2). Mutation frequencies may be enhanced or suppressed by careful selection of the experimental conditions (see Sect. 5).

5. Current Prospects for Radiation Studies on Cultured Plant Cells

From the foregoing discussion it is apparent that much more fundamental work is needed to define the radiosensitivities of cultured plant cells. Recent progress in culturing isolated protoplasts has provided the opportunity to critically evaluate the survival of single plant cells (GALUN and RAVEH, 1975; VARDI *et al.*, 1975) and to relate this information to the known radiosensitivities of the intact plants (see SPARROW *et al.*, 1965). Table 1 summarizes data on various assays which have been employed to evaluate radiosensitivity of cultured plant cells. As emphasized throughout the present discussion, the most sensitive (and most significant) parameter is the reproductive potential of individual cells. This becomes especially apparent when the results from assays of cytoplasmic streaming, growth by weight increase, and growth by colony formation in irradiated tobacco cells are compared (Table 1).

Since cells of different animals vary greatly in their ability to excise DNA lesions (HART and SETLOW, 1974a), it is possible that different plants also vary in DNA repair capacity. A survey of DNA repair in our important crops would point out any individual species which may be more sensitive to environmentally induced DNA damage. Measurements of plant growth may well be incapable of detecting the accumulation of sublethal levels of DNA damage, even though such damage might exert subtle effects on productivity. Increases in solar UV resulting from degradation of the UV-screening stratospheric ozone by pollutants have been predicted (see Sect. 1). The potential biological effects on plants of increased

Table 1. D_{37} values for various end points used in ionizing radiation studies with cultured plant cells[a]

Endpoint assayed	Material	Approximate D_{37}[b] (Krad)	Reference
Cytoplasmic streaming	Microcultured tobacco cells	250–300	CRUZ and HILDEBRANDT (1968)
Cytoplasmic streaming	Microcultured grape cells	0.5–1	ARYA and HILDEBRANDT (1969)
Cell wall regeneration	Wild carrot protoplasts	40	HOWLAND (unpublished data)
Growth (fresh/dry weight)	Tobacco suspension culture	18	VENKETESWARAN and PARTANEN (1966)
Growth (fresh/dry weight)	Bean callus culture	19	BAJAJ *et al.* (1970b)
Growth (cell number)	Pea root explants	3	BURHOLT and VAN'T HOF (1974)
Growth (colony formation)	*Haplopappus*, 1- to 4-cell groups	3	ERIKSSON (1967b)
Growth (colony formation)	Tobacco protoplasts	0.5–1	GALUN and RAVEH (1975)
Growth (colony formation)	*Citrus* protoplasts	4	VARDI *et al.* (1975)
Growth (height)	Tobacco seedlings	3–5	VENKETESWARAN and PARTANEN (1966)
Growth (fresh/dry weight)	Bean seedlings	2	BAJAJ *et al.* (1970b)
Growth (colony formation)	Various cultured mammalian cells	ca. 0.2	SETLOW and SETLOW (1972)

[a] Data for seedlings and for cultured mammalian cells included for comparison.
[b] Values estimated from data in cited reference.

solar UV and other increases in environmental mutagens are largely unknown at present. Radiation studies on plant cells offer a sensitive method with which to obtain this vital information (e.g. HOWLAND, 1975).

Since some plant species can be regenerated from cultured cells, somatic genetics can become an important complement to the standard techniques of the plant breeder (see Sect. 2 and 4.7). Radiation, as well as chemical, mutagenesis can be employed to broaden the relatively limited base of genetic variability in many crops (see CARLSON and POLACCO, 1975).

Both UV and ionizing radiations have been shown to reduce the effective auxin concentration in plant tissues (e.g. KLEIN and VOGEL, 1956; KLEIN, 1968). This approach might be used to advantage in experiments aimed at achieving regeneration of certain cultured plant tissues which do not respond to manipulation of exogenous hormone levels (J. H. YOPP, pers. comm.).

Of the several known systems which can repair DNA lesions, PR (which is specific for pyrimidine dimers) and excision-repair appear to be "accurate", while postreplication repair is "error-prone," resulting in a higher frequency of mutations (see CLEAVER, 1974; TROSKO and HART, 1976). Induced mutation frequency may be controlled to some extent by application of our knowledge of these repair systems. For example, cells undergoing rapid proliferation would be expected to display higher induced mutation frequencies than cells synthesizing DNA at a lower rate. Likewise, agents which inhibit excision-repair will result in enhanced mutation yields [e.g. phorbol myristate acetate (TROSKO and HART, 1976)].

In addition, radiation mutagenesis can be used to dissect normal developmental processes (DULIEU, 1972), as for example, photomorphogenesis in fern gametophytes (HOWLAND and BOYD, 1974).

An efficient system has recently been developed for studying the process of virus infection and development in cultured plant protoplasts (see ZAITLIN and BEACHY, 1974), thus providing a convenient approach to the study of plant virus radiobiology.

These are but a few of the potential applications of radiation studies on cultured plant cells. It is surely true that in coming years we shall see a continued and expanding interest in these powerful approaches to the solution of problems in plant genetics, development, and physiology, and applied problems in agriculture.

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References see page 778.

3. Cryobiology of Plant Cell Cultures and Establishment of Gene-Banks

Y. P. S. BAJAJ and J. REINERT

1. Introduction

In the past, interest in the cryobiology of plants was focused on the preservation of fruits, vegetables and various plant products (JOSLYN, 1966). These studies aimed to store and preserve, or to increase the shelf-life of various materials. However, after the publication of "Life and Death at Low Temperatures" by LUYET and GEHENIO in 1940, there has been an upsurge of interest in the freeze storage of biological materials at super-low temperatures, and plenty of literature has accumulated in the field of cryobiology (see MERYMAN, 1966a; MAZUR, 1970). Most of the information we have today stems from work on animals and their tissue cultures including reports showing the revival of larvae and caterpillars (SCHOLANDER *et al.*, 1953) or whole insects (ASAHINA and AOKI, 1958) from super-low temperatures. The possibility of extension to higher plants and animals is no longer science fiction but a challenge for modern cryobiologists. Suspended animation, prevention, or delay of the ageing process, and indefinite preservation of rare genomes by cryobiological methods are some of the future problems.

Isolated cells and tissue cultures are the ideal system for studying the fundamental aspects of the biology of freezing and the mechanics involved. Cell cultures can be grown in synthetic media and are not dependent on one another for their growth, whereas cells in an organized tissue or an organ are interdependent. In this connection plant cells, unlike animal cells, have the further advantage of totipotency and whole plants can be regenerated from them.

There are only a limited number of reports on the freeze storage of isolated plant cells (Table 1). This article summarizes the literature available, facilities required, technical procedures, problems involved, and emphasizes the prospects of the freeze-storage and establishment of gene banks of plant cells with rare and useful genomes.

2. Technology of Freeze Preservation

The technology for the freeze preservation of plant cell cultures and the eventual regeneration of plants from them, involves the following steps:

1. Raising sterile tissue cultures and cell suspensions
2. Addition of cryoprotective agent
3. Subjecting cell cultures to super-low temperatures by regulated slow rate of cooling or after prefreezing
4. Storage of frozen cells in liquid nitrogen

5. Thawing or rapid rewarming of cells
6. Removal of cryoprotectant by repeated washing
7. Determination of viability
8. Reculture of the retrieved cells
9. Induction of growth and regeneration of plants.

Callus can be induced and grown from almost every part of the plant. The desired explants are first surface sterilized, usually with 1–2% solution of sodium hypochlorite or more conveniently, with 5–10% solution of commercially available detergents such as Bleach. After sterilization they are washed and rinsed 2 or 3 times with sterile distilled water, cut into pieces of suitable size (1 cm^2) and aseptically cultured on agar-solidified medium. The cultures are incubated under controlled conditions at $25 \pm 2°$ C in dark. Basal media of WHITE (1943), and MURASHIGE and SKOOG (1962) supplemented with indoleacetic acid (2 mg/l) or 2,4-dichlorophenoxyacetic acid (1 mg/l) and kinetin (0.5 mg/l) induce quick proliferation in most tissues and yield a sufficient callus mass ready to be subcultured.

The suspension cultures are produced from actively-growing juvenile callus. The callus is removed from the culture tubes and transferred to 125–250 ml Erlenmeyer flasks containing 30–60 ml of liquid medium with low concentrations of auxin and cytokinin. The cultures are then kept on a mechanically agitated shaker. Depending on the texture of the callus, the first suspension of cells is produced within 1–2 weeks. The cell suspension is further filtered through a nylon mesh and subcultured periodically. It is highly desirable that an actively growing cell suspension in an exponential phase containing small cell clumps should be used for the experimentation.

The flasks containing suspensions are transferred to an ice-bath, their contents are allowed to settle, and the excess of medium is pipetted out. One ml samples of the concentrated cell cultures are distributed into sterile plastic ampoules (Fig. 1E), and the extra medium removed. *(It is recommended that sufficient quantity of the cell/unit volume should be taken).* One ml of a suitable cryoprotective agent (usually dimethylsulfoxide 5–10%) is then added to the ampoule in four instalments of about 0.25 ml each at an interval of 5 min. *This gradual addition of the cryoprotectant is very important*, because sudden addition causes plasmolysis of the cells. The suspensions are allowed to remain with the cryoprotectant for about 20–30 min. *During this time it is advisable to maintain the ampoules in an ice bath*, as exposure of cells to the cryoprotectant at a room temperature adversely effects their viability. After addition of the cryoprotectant the ampoules are then subjected to super-low temperatures and frozen. For this purpose, two methods are generally applied: (1) prefreezing, (2) regulated slow rate (1–5° C/min) of cooling. These can be achieved by a number of different types of freezing units, some of which are shown in Figures 1–4. Some are manually controlled, while others are semi- to completely automatic and these various rates of cooling are preset and recorded.

2.1 Freezing Units

Various types of cryostats and freezing units are available by which different rates of cooling can be easily regulated; following are some of the common ones which are being routinely used.

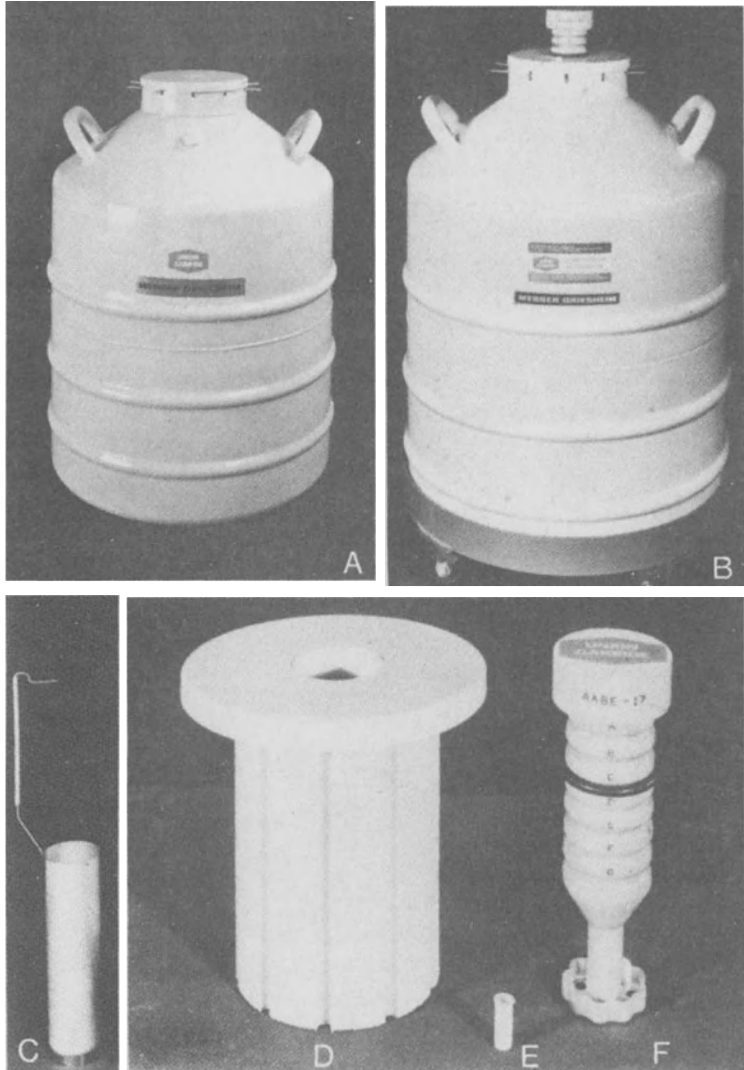

Fig. 1A-F. Freezing Unit LR 33 with Biological Freezer-6 (Union Carbide Corporation—photographs courtesy of Messer Griesheim GmbH, 1-Berlin 12). (For description see text)

2.1.1 LR-33 Biological Freezer-6 (Fig. 1A-F)

This unit (Fig. 1A, B) is manually operated, and biological materials can be frozen at a desired rate of cooling by regulating the plug BF-6 (Fig. 1D, F). The rate of cooling is determined by the number of tubes or ampoules (Fig. 1E) and their distance from the level of liquid nitrogen, which can be controlled by the position of the rubber ring (Fig. 1F). After the controlled freezing, the ampoules are stored in containers (Fig. 1C) of the freezing unit. With this unit up to 8 ampoules can be frozen at one time.

Fig. 2. Programed Freezer R 201 (Photograph Courtesy of Messers GV Planer Ltd., Sunbury-on-Thames, England). (For description see text)

2.1.2 Programed Freezer R 201 (Fig. 2)

This freezing unit provides a compact means for the programed cooling of biological specimens prior to storage. The operation relies on the controlled injection of atomized liquid nitrogen into the work chamber. The temperature is monitored with the use of a resistance thermometer situated in the large capacity 28 l chamber, a chart recorder providing a permanent record of the cycle. A special feature of the equipment is a thermal pump utilized to eject the refrigerant from the Dewar container, affording a high degree of reliability. The liquid nitrogen content of the container is continuously monitored during the operation of the equipment by weight determination of the container.

2.1.3 Mini-Freezer R 202 (Fig. 3)

This equipment comprises a vacuum insulated vessel of about 4 l volume which may be cooled from ambient to -170° C at a predetermined rate by controlled

Fig. 3. Programed Mini-Freezer R 202 (Photograph Courtesy of Messers GV Planer Ltd., Sunbury-on-Thames, England). (For description see text)

injection of gaseous liquid nitrogen into the chamber. A built-in heater system allows rapid reversal of the cooling process when necessary. Up to 60 glasses or plastic ampoules mounted in canes may be processed at any one time. The cooling chamber is mounted on a transport trolley in which is also mounted the electronic controller. With this unit various rates of cooling ranging from 0.1 to 10° C/min can be linearly fixed, however, the preset program rate can be overriden manually at any time.

The cultures subjected to ultracooling can be stored at −196° C in the liquid nitrogen containers for various lengths of time, and can be taken out and thawed when required. *Rapid rewarming of the cultures in a water-bath at 35–40° C is recommended.* To reduce the rate of metabolism it is is desirable that between various treatments the cultures be maintained in a bucket of ice. The thawed cultures are centrifuged, the cryoprotectant pipetted out and the *cells washed at least three times* with the appropriate medium to remove any traces of cryoprotectant that might have been left. The cells are then recultured in 100 ml flasks

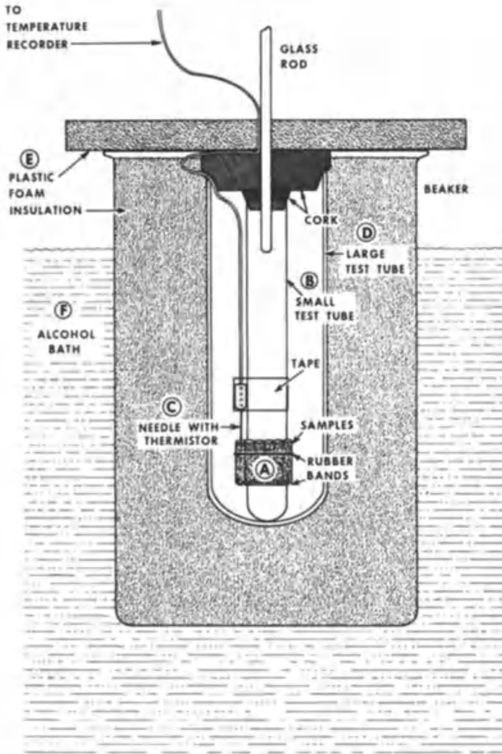

Fig. 4. Apparatus used for freezing beet root segments. The thickness of plastic foam insulation in the beaker determines the rate of heat loss to the cold alcohol bath. (Courtesy of Dr. B. J. Finkle, 1974)

(containing 25 ml of the medium) and maintained on the rotary shaker, or plated directly in the agar-solidified medium. By manipulation of various nutritional and physical conditions, morphogenesis is then induced in these cultures.

2.2 Determination of Cell Survival

The viability of the frozen cells can be determined by the following methods:

1. Fluorescein Diacetate Staining. This method is based upon the fact (WIDHOLM, 1972a) that living cells stained with fluorescein diacetate show fluorescence under UV light, while the dead cells do not (Fig. 5A–D, 6C, D). In this method, first a drop of 0.1% fluorescein diacetate is mixed with a drop of cell suspension and allowed to stand for 5 min. They are observed under the tungsten light and the number of free cells or cell aggregates is counted in one field. The light source is then changed to UV and the %age of cell survival is estimated by the number of cells showing fluorescence. It has been our experience that free isolated cells are normally not able to withstand freezing while the cell clumps and aggregates survive. In such cases, especially in large aggregates where it is sometimes not possible to count the number of cells, it is advisable to score the aggregate as a unit and the percentage survival could then be expressed in terms of number of aggregates.

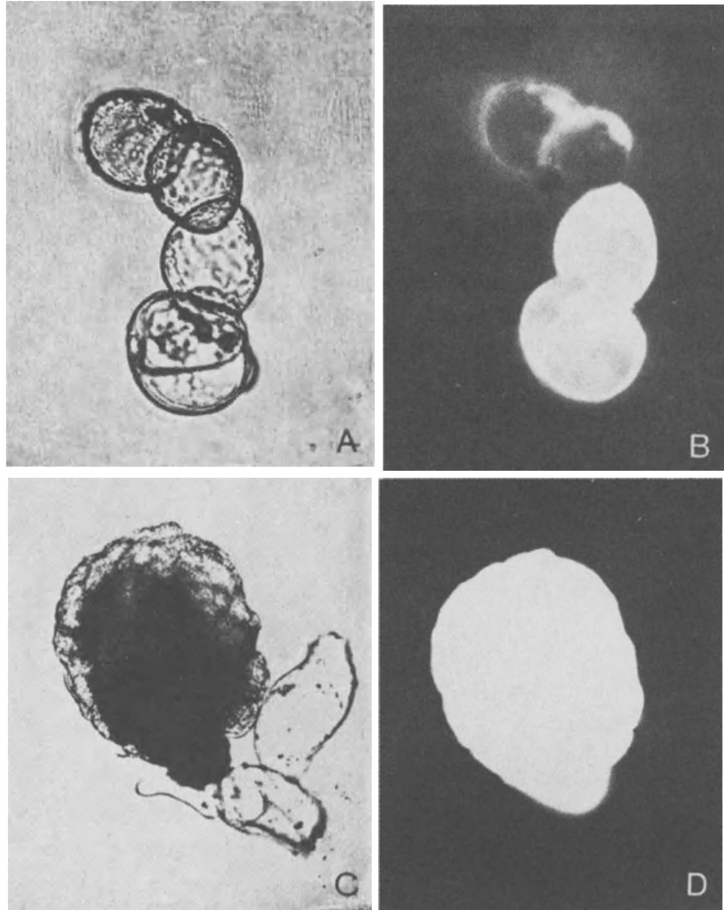

Fig. 5A-D. Revival of carrot cell suspensions subjected to super-low temperatures, and stained with fluorescein diacetate. (A) A filament of cells (in tungsten light) taken from a culture frozen at $-20°$ C for a week. (B) Same, in UV light; note the partial revival of the filament. (C, D) A proembryo along with free cells subjected to $-20°$ C (1 h) and $-70°$ C (30 min), and then immersed in liquid nitrogen (cryoprotectant DMSO 5%, thawing at 37° C; note the complete revival of the proembryo, and the death of single cells. (After BAJAJ, 1976)

2. *Triphenyl Tetrazolium Chloride Method.* In this method cell survival is estimated by the amount of formazan produced as a result of reduction of triphenyl tetrazolium chloride (TTC) (STEPONKUS and LANPHEAR, 1967) which gives a pink color. The procedure involves the following steps:

1. Buffer solution: 78% $Na_2HPO_4 \cdot 2H_2O$ solution: 0.05 M (8.9 g/l), 22% KH_2PO_4 solution 0.05 M (6.8 g/l).
2. TTC solution: 0.6% TTC dissolved in buffer solution.
3. About 150 mg of cell sample is put into 3 ml of TTC solution and incubated for 15 h at 30° C.
4. The TTC solution is drained off and the cells washed with distilled water.

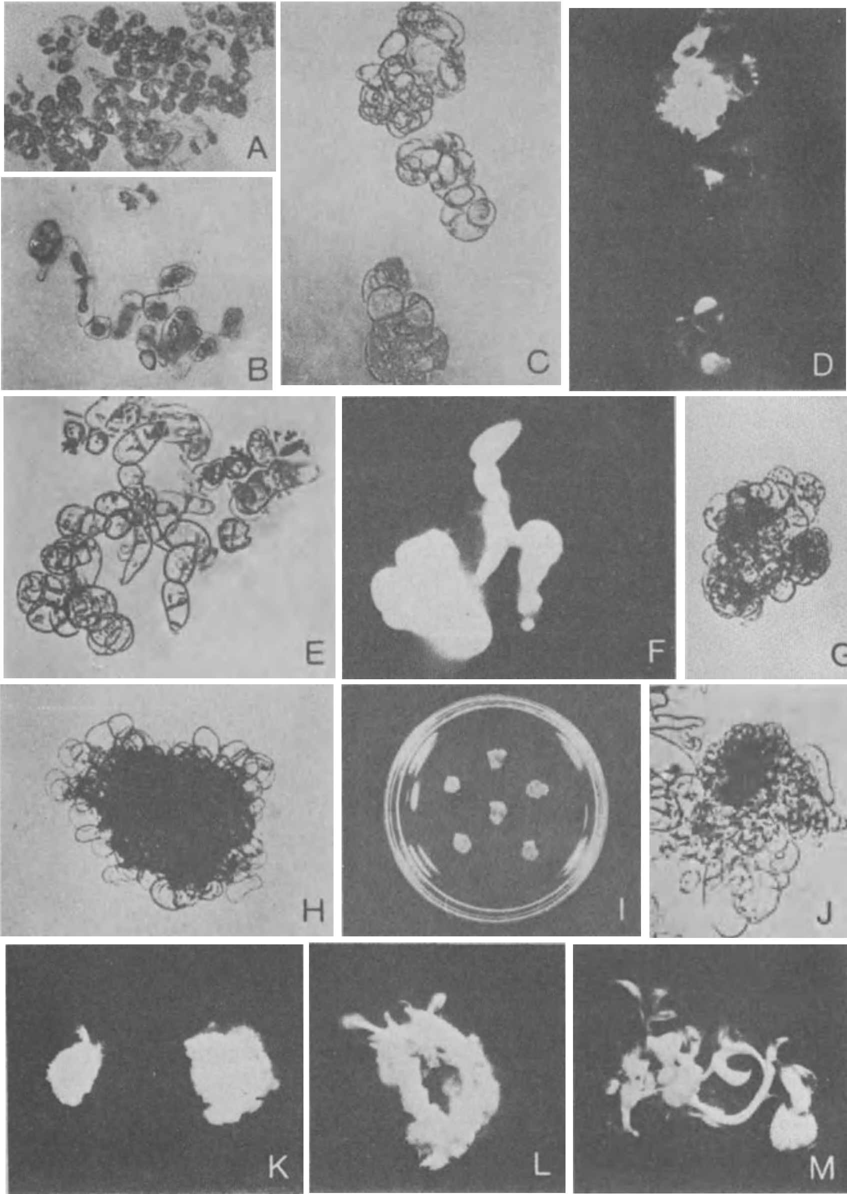

Fig. 6A-M. Regeneration of plantlets from cell suspensions of *N. tabacum* subjected to super-low temperatures. (A, B) Cell suspension before (A) and after freezing (B) in liquid nitrogen; note the freezing injury in the free cell. (C, D) Clumps of cells subjected to $-196°$ C in the presence of DMSO 7%; note the partial survival of the clumps in (D) as shown by ultraviolet fluorescence with fluorescein diacetate staining (DMSO 7%, thawing at 37° C). (E, F) Cell suspensions maintained at $-20°$ C for a week, note the ultraviolet fluorescence of the clumps and the filament of cells. (G-I) Various stages in the formation of colonies and callus obtained from frozen cells. The growth in colony (G, H) formation was visible after a lag period of 3–4 months. (J) An actively growing meristemoidal group of cells. (K) Masses of callus obtained from Figure I on MS medium supplemented with IAA (2 mg/l) and kinetin (0.05 mg/l). (L, M) Stages in the differentiation of callus into shoots (L) and plantlets (M) on MS medium fortified with IAA (2 mg/l) + kinetin (2 mg/l). (From BAJAJ, 1976)

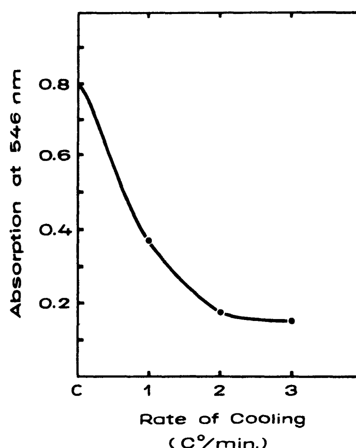

Fig. 7. Effect of various rates of cooling on tetrazolium reduction in *Datura* cell clumps frozen at −196° C in the presence of 7% DMSO. The absorption at 546 nm was taken as the criterion for cell survival. *c*: control. (After BAJAJ, 1976)

5. Cells are centrifuged and extracted with 7 ml of ethanol (95%) in a water-bath at 80° C for 5 min.
6. The extract is cooled and made to 10 ml volume with 95% ethanol.
7. The absorbance (pink color) is then recorded with a spectro-photometer at 530 mμ.

The amount of formazan produced by the frozen cells is expressed as a percentage (survival) of formazan produced by the control cell suspensions (Fig. 7).

3. *Growth Measurements.* By employing various parameters i.e. mitotic index, cell number, cell culture volume, dry and fresh weights and plating efficiency, the growth of frozen cultures can be compared with the control.

The staining method alone may not be sufficient and satisfactory in determining the viability of the cells, as sometimes it is not possible to decide whether the cell is living or dead when there is partial staining or light fluorescence. Such cells showing a weak staining reaction might eventually die in cultures. Furthermore, there are cells which show partial staining immediately after cold treatment but die soon afterwards in cultures. Some cells may go undetected but later in culture they continue to grow and divide. There are also cells which are partially injured or in a state of cold-shock, which do not divide but retain some physiological activity i.e. respiratory and enzymatic. Such cultures with low survival rate often show a long lag phase, which could perhaps be the time to repair the cell damage.

The staining procedure would perhaps be more suitable for somewhat resistant strains showing high viability, but for highly susceptible cell cultures where the percentage of cell survival is very low, there is the likelihood that partially living cells may not be detected. In this connection the refined technique of triphenyl tetrazolium chloride for determining the cold injury to the plant seems to be more promising, though not foolproof.

An ideal method for determining the viability of cells and tissues must enable one to ascertain if samples which show a positive reaction at the time of testing indicate future viability (STEPONKUS and LANPHEAR, 1967).

Any data on the viability of cells based on staining reaction alone would be insufficient and misleading. In some of our experiments where frozen tobacco

(*Nicotiana tabacum*) cell clumps gave negative viability response, cultures started to grow and divide after a lag period of 4–6 months (BAJAJ, 1976). ARPAI (1963) reported that *Pseudomonas fluorescens* lag phase is directly proportional to the "metabolically" injured cells (MAZUR, 1966). The long lag phase could be due to (1) the cold-shocked cells and the partially damaged cells needing time to revive and repair, and (2) the cryoprotectant suppressing the growth.

3. Factors Influencing Revival of Frozen Cells

From the literature accumulated so far, it has emerged that the degree of success for the revival of plant cells subjected to super-low temperatures depends on a number of factors—the most critical being: (1) age, nature and density of cells; (2) cryoprotective agent; (3) rate of cooling; (4) method of thawing; and (5) storage temperature.

3.1 Age, Nature and Density of the Cells

The physiological state of the plant cell cultures considerably influences their reaction to cooling, and the extent of injury caused depends upon, (1) *meristematic cells:* periodically transferring and actively growing young suspensions of cells in an exponential phase is much better than older cultures containing large and thick-walled cells with scanty cytoplasm. Highly vacuolated cells are easily killed due to accumulation and bursting of ice crystals within the vacuoles, (2) *water contents*: the higher the water content of a cell or tissue the more susceptible it is to freezing injury; spores and seeds are easy to store and preserve. Plantlets with low freezable water can withstand extremely low temperature (LUYET and GEHENIO, 1940), as the bound water in the cells does not freeze. SUN (1958) reported that young pea (*Pisum sativum*) seedlings, in which the moisture content was lowered to 27–40%, could withstand liquid nitrogen but that in older seedlings only the stem-tip survived. This is interesting as the stem-tip cells are nonvacuolated and thin-walled, and are thus closer to the actively growing cell suspensions. SAKAI (1960) observed that if mulberry (*Morus bombycis*) twig cells were dehydrated sufficiently by extracellular freezing (prefreezing) they could survive immersion in nitrogen, (3) *single cells vs clumps:* filtered cell suspensions of free cells occasionally survive while actively growing suspensions with clumps; small colonies of cells with highly cytoplasmic cells show high (70%) survival in carrot (BAJAJ and REINERT, 1975). However, large masses of callus stored in liquid nitrogen became spongy and died when thawed, possibly due to the formation of intervellular ice crystals which force the air out, (4) *density of cells:* the density of the cells or biomass per ampoule (with relation to volume of the medium) is an important criterion for cell survival. The ampoules containing thick and packed suspensions often show higher cell survival compared to those with lower cell density (BROWN *et al.*, 1974; NAG and STREET, 1975b; BAJAJ, 1976), (5) *degree of cold hardiness:* the sensitivity of cells to cold might

also depend on the degree of frost hardiness of a plant, for instance, cells from a frost-resistant woody tree with higher amounts of sugars and polyhydric alcohols (SAKAI *et al.*, 1968) might react differentially to super-low temperatures.

3.2 Cryoprotective Agents

POLGE *et al.* (1949) first reported the protective effect of glycerine on animal cells subjected to low temperatures. Since then a large variety of substances have been shown to possess cryoprotective properties; some of which are dimethylsulfoxide (LOVELOCK and BISHOP, 1959), ethylene glycol (SAKAI, 1960), diethylene glycol, propylene glycol, hexamethylene tetraime, pyridine N-oxide acetamine, dimethylacetamide (VOS and KAALEN, 1965), acetamide and glyceryl monoacetate (LOVELOCK, 1954), polyvinylpyrolidone (WHITTINGHAM *et al.*, 1972) and various sugars. Among these agents dimethylsulfoxide (DMSO) has been extensively used and has proved to be an excellent cryoprotectant. An efficient cryoprotective agent should: (1) have low molecular weight, (2) be easily miscible with the solvent, (3) be nontoxic even at low concentrations, (4) be easily washable from the cells, and above all (5) permeate rapidly into the cells. All these criteria are fulfilled by DMSO. However, at higher concentrations it reduces respiration and inhibits RNA and protein synthesis in isolated plant cells and tissues (BAJAJ *et al.*, 1970a), so optimal concentrations should be employed. In our work on the effect of various concentrations (5, 7, and 10%) of DMSO (without freezing) at different temperatures showed that 5–10% did not significantly affect the cell viability up to 1 h at 3° C, while at 23° C the rate of survival continued to drop with the passage of time. Figure 8 shows the effect of DMSO on carrot cell cultures. An almost similar trend has been observed with *Datura stramonium* cell clumps. NAG and STREET (1975a) from their study of the effect of various cryoprotectants concluded that 5% DMSO proved to be most effective with carrot and *Atropa belladonna*, while 10% glycerol was optimum for sycamore cells. Up to 70%

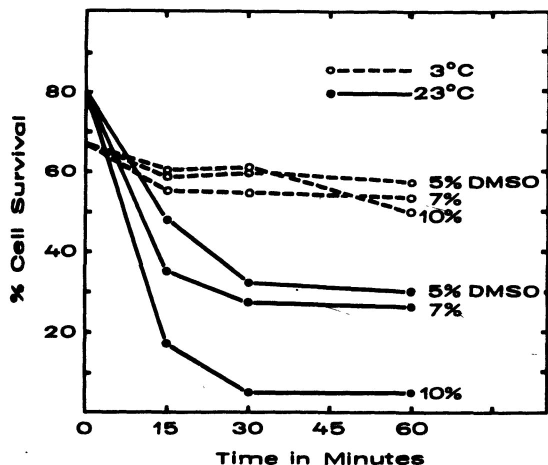

Fig. 8. Effect of various concentrations of DMSO (5,7 and 10%) on survival of carrot cell suspensions, pretreated in ice (about 3° C) and at room temperature (23° C). Survival was determined by the FDA staining. (After BAJAJ and REINERT, unpublished)

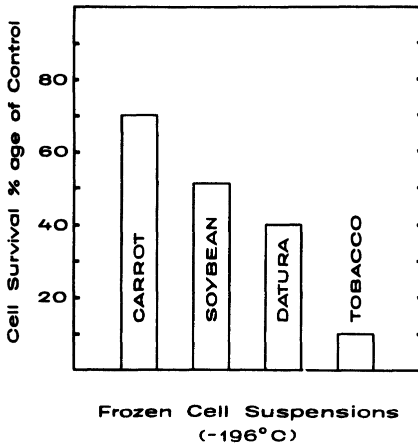

Fig. 9. Histogram showing the percentage survival (as determined by fluorescein diacetate staining) of cell suspensions of carrot. soybean, datura and haploid tobacco subjected to −196° C and cooled at 2° C/min in the presence of 5% DMSO. (After BAJAJ, 1976)

carrot cells survived at −196° C (BAJAJ, 1976) in the presence of 5% DMSO, while in soybean, *Datura* and haploid tobacco suspensions, cells survival was 52, 40, and 10% respectively (Fig. 9).

The optimal concentrations of various cryoprotectants used for freeze preservation of plant tissue cultures are summarized in Table 1. It is amazing to note that SUGAWARA and SAKAI (1974) used tremendously high concentrations of DMSO (24%) combined with glucose (10%) for sycamore cells, and still obtained 20–30% cell survival. In our opinion this high concentration of cryoprotectants would certainly plasmolyse and considerably damage the cells and should be avoided.

Workers have also used combinations of two or more cryoprotectants to obtain positive results. LATTA (1971), for instance, could not successfully revive *Impomoea* cells with only 5% DMSO, while with a mixture of DMSO (2.5%), glycerol (2.5%) and sucrose (6.5%), the frozen cells revived and resumed growth.

Table 1. Summary of the optimal conditions for the survival of plant cell cultures subjected to super-low temperatures

Plant species	Cryoprotectant	Cooling	Thawing	Results	Reference
Acer pseudoplatanus	DMSO 24% + glucose 10%	Pre-freezing −30 to −50° C and then at −196° C	40° C	Cells showed 20–30% TTC reduction	SUGAWARA and SAKAI (1974)
	Glycerol 10%	at 1° C/min	37° C	28% cell survival	NAG and STREET (1975b)
	DMSO 15%	at 1–3° C/min	30° C	20% cell survival	HENSHAW (1975)
Atropa belladonna	DMSO 5%	at 2° C/min	37° C	40% cell survival	NAG and STREET (1975a, b)

Table 1 (continued)

Plant species	Cryoprotectant	Cooling	Thawing	Results	Reference
Chrysanthemum morifolium	DMSO 5% or Sucrose 10%	−3.5° C −5.5° C	27° C	Low survival	BANNER and STEPONKUS (1972)
Datura stramonium	DMSO 5%	Cooling at 1–2° C/min till −100° C and then at −196° C	37° C	40% Cell survival	BAJAJ (1976)
Daucus carota	DMSO 10% or Glycerol 5%	at 2–4° C/min stored at −196° C	37° C	Cells survived 2 months storage	LATTA (1971)
	DMSO 5%	at 2° C/min stored at −196° C	37° C	65% cell survival, plant regeneration	NAG and STREET (1973)
	DMSO 5–10%	at 1–2° C/min −70° C −196° C	30° C and room temp.	Embryogenesis	DOUGALL and WETHERELL (1974)
	DMSO 5–7%	Pre-freezing at −20° C and −70° C, or cooling at 2° C till −100° C and stored at −196° C	37° C	70% survival, plants regenerated	BAJAJ and REINERT (1975), BAJAJ (1976)
Glycine max	DMSO 7%	same	same	52% survival	BAJAJ (1976)
Ipomoea sp.	DMSO 2.5% + Glycerol 2.5% + Sucrose 6.5%	at 2–4° C/min −196° C	same	Heavy growth in 10 days	LATTA (1971)
Linum usitatissimum	DMSO 10%	at 5–10° C/min −50° C	40° C	14% cell survival	QUATRANO (1968)
Nicotiana tabacum (haploid)	DMSO 5%	Pre-freezing at −20, −70° C or at 1–2° C/min, −196° C	37° C	Long lag phase, 5–10% survival, plants regenerated	BAJAJ and REINERT (1975), BAJAJ (1976)
Populus euramericana	—	Pre-freezing −30°, −70° C, −120° C and then at −196° C	slow warming in air	Callus masses survived	SAKAI and SUGAWARA (1973)
Prunus cerasus	10–20% sucrose medium	Hardened at 2° C, and subjected to subfreezing temperature	—	Callus showed resistance to −30° C	TUMANOV *et al.* (1968)

Likewise, NAG and STREET (1973) observed a slight increase in carrot cell survival in the presence of a mixture of DMSO (5%) and glycerol (10%), than when glycerol (10%) was used alone. For future work it might be beneficial to employ a mixture of several cryoprotectants, especially in cases where DMSO or glycerol alone do not protect the cells sufficiently. A glance at Table 1 shows that the concentration of the cryoprotectant varies with the plant species, some of which are more sensitive than others. Although a study of the literature gives a general idea about the range of concentration of the cryoprotectant, depending on the nature of the plant material (hardy vs nonhardy), the type and concentration of the cryoprotectant will also have to be studied.

Various interpretations have been given with regard to the mechanism of the action of cryoprotectants. To deal with these aspects in detail is beyond the scope of this article, however, the detailed chemistry and mechanism, and biochemical aspects of these additives have been discussed by various workers (NASH, 1966; ROWE, 1966). The clear cut basis for the action of the cryoprotectant remains obscure, however, they have been shown to act as antifreeze agents by reducing the concentration of intracellular salts (LOVELOCK, 1953) and the formation of ice crystals. Depending upon the nature of the cryoprotectant it could prevent the immediate freeze or storage injury, or both. In microorganisms it is suggested (MAZUR, 1966) that storage death results from long-term exposure to residual concentrated solutions, and a cryoprotectant could reduce the decay rate either by acting as an innocuous diluent of the toxic solutes or by actually blocking or slowing their deleterious action.

According to the sulfhydryl-disulfide hypothesis (LEVITT, 1962) dehydration of the cells, and thus frost injury, is induced by intermolecular SS formation, and the cryoprotectants prevent or inhibit the formation of these SS bonds (ANDREWS and LEVITT, 1967).

ROWE (1966) suggested that cryoprotectants interact directly or indirectly with the cell membrane to stabilize the water-lipid-protein complex tertiary structure. It is at the cellular membranes that biological integrity appears to be insulted by freezing, so it is at the membrane level that biochemical understanding of cryoprotectants must be sought.

3.3 Cooling

The rate of cooling and the method of thawing are the two most critical parameters in determining the extent of success in revival of the cells. Faulty cooling may affect the cells in a number of ways, i.e., direct lowering of the temperature may interfere with the biochemical and physical pathways (MERYMAN, 1966b), extra and intracellular formation of ice crystals and the changes in the solutes of the cells which lead to dehydration. The formation of intracellular ice crystals is one of the main causes of freezing injury and is usually lethal. To prevent this injury, cooling must be slow enough to let all freezable water flow out of the cell during cooling (MAZUR, 1970).

Cooling of cells, as mentioned earlier, can be achieved by prefreezing, and the gradual regulated rates. Table 1 summarizes various methods and the rate of cooling employed for successful revival of plant cell cultures.

Prefreezing involves cooling in two steps, and is effective in preventing and decreasing the intracellular formation of ice crystals. According to SAKAI (1965) "there is a definite temperature at which almost all the easily freezable water in a cell may be withdrawn from the cell by extracellular freezing, and that cells and tissues in this state are not injured, even when exposed to extremely low temperatures". According to SAKAI, this temperature is around −30° C, but may vary with the degree of frost-hardiness of the plant. It was observed (SAKAI, 1960) that when mulberry *(Morus bombycis)* twigs were prefrozen at −30° C, and then subjected to liquid nitrogen, they could be stored for months, while in nature, a temperature below −30° C would kill them. At this temperature easily freezable water is almost entirely removed from the cell (partial dehydration) by the formation of extracellular ice.

By gradual prefreezing (at 3-min intervals at −15, −23, −30, −40, −50, and −70° C) up to 30% of the sycamore *(Acer pseudoplatanus)* cell suspensions could withstand −196° C followed by rapid warming at 40° C (SUGAWARA and SAKAI, 1974). Likewise poplar *(Populus euramericana)* callus first cooled to preconditioning temperatures, and then subjected to −196° C by the prefreezing method could be revived (SAKAI and SUGAWARA, 1973). These workers claimed to have induced an increase in hardiness in such a callus. In view of the economic importance of this plant species, it would be of great benefit if the plants regenerated from such calli also exhibited increased hardiness to frost as well. Tobacco and carrot cells were revived (BAJAJ, 1976) by freezing them first at −20° C and −70° C and then subjecting them to −196° C (Table 2) however, when directly immersed in liquid nitrogen, the cells were inavariably killed.

Earlier work on suspensions of unicellular algae (LEIBO and JONES, 1963; HWANG and HORNELAND, 1965) showed that these cells could be revived when cooled at the rate of 1° C/min, while rapid cooling (20° C/min.) was injurious. Likewise a glance at Table 1 shows that in almost all the plant cell cultures successfully revived, a slow rate of cooling has been employed, this varies between 1° C to 5° C/min., though optimum appears to be around 2° C/min. An idealized scheme of cooling which includes the liquid phase, transition area, and solid phase, has been given in Figure 10. The shape of the curve is a sum of the transient heat-transfer relationship between the specimen and its environment. According to ROWE (1966) maximum survival is obtained by controlling the shape of the curve. Rapid cooling followed by warming, especially slow warming, causes the recrystallization of ice. The studies on mulberry leaves and marrow stem-cells (MAZUR, 1966; SAKAI and YOSHIDA, 1967; MAZUR and SCHMIDT, 1968) suggest that death of the rapidly cooled cells is caused by the growth of intracellular ice crystals rather than their initial formation. The injury of the cells could be due to pressure exerted by the recrystallizing ice crystals which causes the rupture of plasma membrane of the organelles (MAZUR, 1966, 1970), and this stress resulting from intracellular ice can be electronically measured (OLIEN, 1961). However, mechanical injury by freezing does not appear to be great. TRUMP *et al.* (1965) observed that although extracellular freezing produced distortions, the structural resolution after thawing was perfect. In plant tissues (LEVITT, 1966) there is a similar situation in which an extracellular freezing causes a reversible loss of water in the cells. MAZUR (1970) emphasized the close relationship between the

Table 2. The effect of various cold treatments on cell suspensions of *Daucus carota* and *Nicotiana tabacum*. The cultures were cooled in the presence of 5 or 7% DMSO and thawed at 37° C in a water-bath. The percent growth increase is represented by + (10–20%), + + (20–30%), + + + (30–50%), + + + + (50–70%). (From BAJAJ, 1976)

Plant species	Temperature	Method of cooling	Duration of storage	Growth response	Lag period
Daucus carota	1. −20° C	Direct exposure	4 h	+ + + +	3 wks
	2. −20° C		24 h	+ + +	3–4 wks
	3. −20° C		1 wk	+ + +	3–4 wks
	4. −20° C		2 months	+ +	8–9 wks
	5. −70° C		2 h	+ + +	4–5 wks
	6. −20° C −70° C.	Direct exposure	2 h 1 h	+ + +	4–5 wks
	7. −20° C −70° C −196° C	Pre-freezing	1 h 30 min 2 min	+ + +	4–5 wks
	8. −196° C	Slow cooling at 2° C/min	3 months	+ + + +	4–5 wks
Nicotiana tabacum	1. −20° C	Direct exposure	24 h	+ + +	5 wks
	2. −20° C		1 wk	+ + +	7 wks
	3. −20° C		1 month	+ +	7–8 wks
	4. −20° C		2 months	+	7–8 wks
	5. −196° C	Slow cooling at 2° C/min	1 month	+	3–4 months
	6. −196° C		4 months	+	3–4 months
	7. −196° C		6 months	+	3–4 months

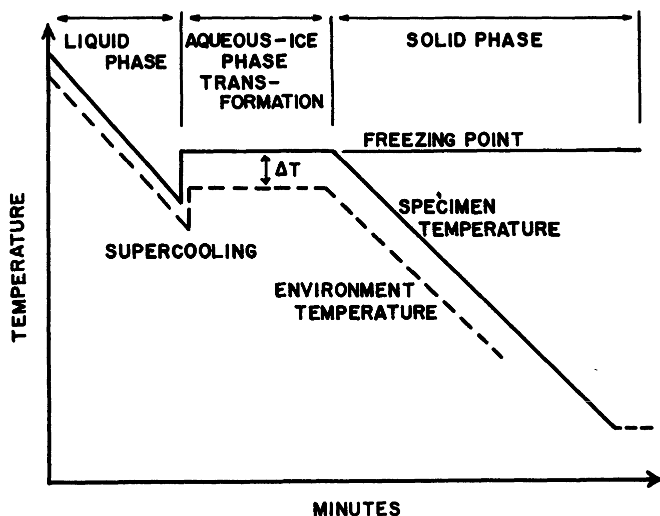

Fig. 10. An idealized cooling diagram illustrating the phases of transient heat transfer relationships between the biological specimen and the cold environment. (Courtesy of Dr. A. W. ROWE, 1966)

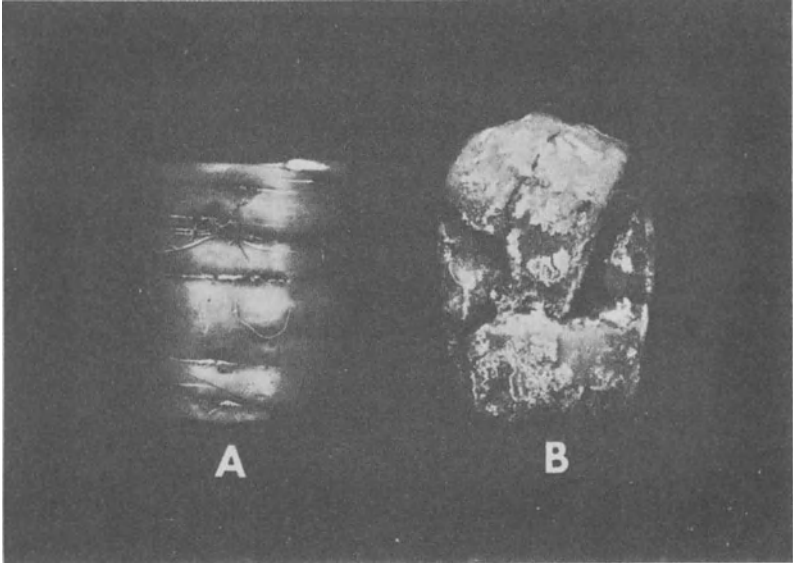

Fig. 11A and B. Thick root-segments of carrot, (A) control, (B) frozen: Rapid freezing and storage at $-20°$ C caused them to crack and break (B). (After BAJAJ and REINERT — unpublished)

responses of cells to freezing and the properties of cell membranes. According to him, membranes are the chief targets of freezing damage as shown by the studies on mito chondria and chloroplasts (HEBER, 1967, 1968).

SAKAI *et al.* (1968) reported the successful revival of cells of mulberry by rapid cooling and rewarming, but they attributed this to the highly resistant nature of the woody plant, and to the presence of high contents of sugar and polyhydric alcohol which contribute to the prevention of injurious intracellular crystalization which occurs during rapid cooling. In fact spontaneous exposure of the large plant tissues or organs to rapid freezing could be very injurious as there is hardening of the surface before freezing and then an expansion of the interior portion. In our studies (BAJAJ and REINERT, unpublished) rapid freezing and storage of carrot roots or their thick segments at $-196°$ C caused them to crack (Fig. 11A, B) or even break into small pieces. So in subsequent experiments we preferred to use either relatively small segments from the large and old roots or alternatively, very young and small roots were employed. FINKLE *et al.* (1974) observed that small cylinders of *Beta vulgaris* roots, frozen at ultra-slow cooling [0.2° C/h to $-4°$ C (Fig. 4)] produced little damage, as determined by softening and leakage of pigment and electrolytes, however, all of these increased at a faster rate of cooling.

To explain the mechanism of cold or frost injury, various theories have been proposed from time to time. To discuss these in detail is beyond the scope of this article, however, the following are some which have been critically discussed by LEVITT (1966) and MAZUR (1969): (1) Armchair Theories of historical interest, (2) Frost Precipitation Theory, (3) Iljin's Mechanical Stress Theory, (4) Intracellular Freezing Theory, and (5) Sulfhydryl-Disulfide Theory. No single

theory is self sufficient, however, the last one seems to provide logical explanations and could at present be regarded as the working hypothesis. According to this concept (LEVITT, 1962) the dehydration of cells and thus frost injury is induced by the formation of intermolecular disulfide (SS) bonds. As LEVITT (1966) put it: "Frost injury is due to the unfolding and therefore the denaturation of the protoplasmic proteins. This results from the mutual approach of the protein molecules (due to their dehydration during freezing) until they are close enough for the formation of intermolecular SS bonds either by oxidation of two adjacent SH groups or by SH $\leftrightarrows$ SS interchange". According to LEVITT frost resistance is thus due to the resistance against the formation of intermolecular SS bonds.

To summarize, the freezing injury could be due to a number of interdependent factors, i.e., intracellular freezing, dehydration of the cells, increase in the concentrations of solutes (LOVELOCK, 1953), formation of disulfide bonds, and also due to mechanical stress and rupture of the organelles membrane.

3.4 Thawing

The rates of warming considerably influence the LT_{50} of the cells (Levitt, 1966). Slow warming of animal cells has in many instances, proved to be better (MERYMAN, 1966b), while a reverse situation seems to occur in the plant cells, which could possibly be due to the presence of a thick wall. The results obtained by various workers on the rate of warming of plant cells are rather contradictory. For instance, PARKER (1960) observed that Pine leaves cooled to $-45°$ C survived when thawed at the rate of 5° C/h, but died while rewarming at the rate of 10° C/h. On the contrary SAKAI (1971) reported that when unmounted cortical cells of mulberry exposed to liquid nitrogen were rapidly warmed at 40° C all the cells survived, while none survived in the sections rewarmed slowly in the air.

DOUGALL and WETHERELL (1974) observed no difference in carrot cell suspensions between the cells thawed at room temperature and at 30° C; in sycamore cells (SUGAWARA and SAKAI, 1974) likewise no difference in TTC reduction was observed in those thawed at 40° C or in air at 0° C. On the other hand beneficial effect of rapid thawing at 37° C (Table 3) has been emphasized by NAG and STREET (1975b), and BAJAJ (1976). Figure 12 shows the pattern of temperature rise in the ampoules subjected to various methods of thawing, and confirms the beneficial effect of rapid thawing. Although more data are needed on this aspect of the cryobiology of plant cell cultures, at present rapid warming is recommended, because by rapid warming the ice crystals present in the cells melt before they have the opportunity to recrystalize or grow—which generally happens as a result of slow warming.

3.5 Storage Temperature

The injury caused to the cells could be due to freezing or storage the latter occurs primarily when the cells are not stored at sufficiently low temperatures.

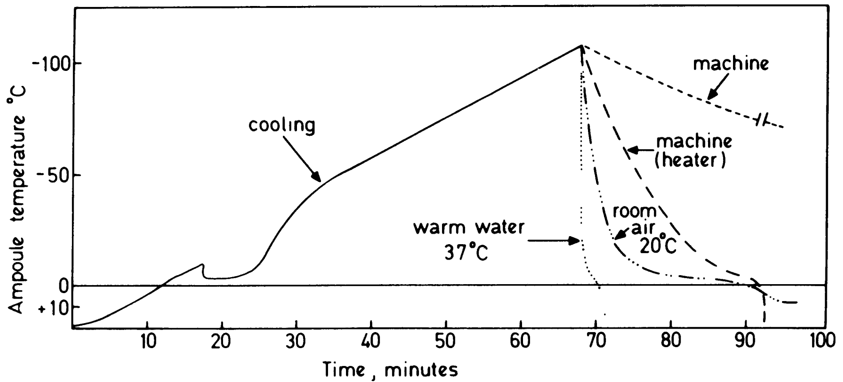

Fig. 12. Progress of the rise in temperature under different thawing conditions. Diploid wild carrot suspension (in medium plus 5% DMSO) frozen to −108° C at 2° C/min before thawing. Machine = slow thawing in programed freezer. Machine (heater) = thawing in programed freezer with heater on. (Courtesy of H. E. STREET, reproduced with permission from Physiol. Plantarum **34**, 261–265, 1975b)

Table 3. Interaction of cooling and thawing rate assessed by percentage cell survival. Cooling rate 2° C/min, to temperatures indicated, followed by immediate transfer to liquid N_2 (−196° C); storage for 16 h at this temperature; followed by recovery at two thawing rates (air at 20° C and water bath at 37° C). (Courtesy of H.E. STREET, reproduced with permission from Physiol. Plantarum **34**, 261–265, 1975b)

Cell line	Temperature reached at 2° C/min before transfer to −196° C					
	−40° C		−70° C		−100° C	
	air (20° C)	bath (37° C)	air (20° C)	bath (37° C)	air (20° C)	bath (37° C)
Carrot (wild, diploid)	5	45	35	55	40	58
Belladonna	2	15	5	18	8	18

The temperature should be sufficiently low for long-term storage of cells to stop all metabolic activity and prevent biochemical injury.

Most tobacco cells frozen for a week at −20° C or for 48 h at −70° C revived and resumed normal growth; however, when stored at −196° C for six months, there was a long lag phase (Table 2) and very few cells survived (BAJAJ, 1976). SUGAWARA and SAKAI (1974) reported that tobacco cell suspensions could withstand −30° C, but not −196° C. On the contrary in our studies the cells survived in liquid nitrogen, though the survival rate was very low. This low survival is, however, attributed to nonoptimal cooling and thawing regimes, rather than storage temperatures. With refinement in technique, there is reason to believe that survival rate can be increased.

At present no data on the comparative effect of various low temperatures on storage of plant tissue cultures is available, but based on our knowledge of animal cells, it is generally believed and agreed that −20° C is not adequate for long-term

storage. Liquid nitrogen does not impair organogenesis (Fig. 6) in tobacco (BAJAJ, 1976), or embryogenic potential in carrot cells (NAG and STREET, 1973; BAJAJ and REINERT, 1975), but the possibility that certain biochemical changes might take place is not completely ruled out. Certain reports on animal cell cultures show that, even at −196° C, some of the enzymes, e.g. aldolases, may lose activity, or show changes in relative proportions of lactic acid dehydrogenase isoenzyme (PETERSON and STULBERG, 1964). Thus, the possibility exists that even at −196° C the migration of water in the frozen cells does take place.

4. Prospects

Because of the morphogenetic potential of the plant cell, the cryobiology of plant tissue cultures offers a wide range of prospects; some of the most obvious follow.

4.1 Conservation of Genetic Uniformity

It has been repeatedly observed that plant cells in cultures show a variety of genetic diversity and variability (PARTANEN, 1963; D'AMATO, 1965; HEINZ and MEE, 1971; BAYLISS, 1973; Chap. III.1 of this Vol.). This variability depends on the origin and the genetic constitution of the tissue, and is considerably influenced by the physical conditions and growth regulators which cause chromosomal instability. After many cultures cells have a tendency to undergo endomitosis, with the result that cultures of various ploidy levels are obtained. Cultured mammalian cell lines have been preserved in defined media and the culture banks of certified animal cell lines have been established (HAUSCHKA *et al.*, 1959). Such an establishment of gene banks for the preservation of genetic uniformity of plant cells, especially for the plants which can be propagated vegetatively by cell cultures is highly desirable. This would ensure the genetic uniformity of the material.

4.2 Preservation of Rare-Genomes

Various genetic variabilities (i.e. chromosomal aberration, change in the level of ploidy and mutations) are a common trait in long-term tissue cultures (DULIEU, 1972). These phenomena, though undesirable for maintaining the uniformity and stability of the clones, can nevertheless be exploited by the plant breeders and geneticists. Those variables, which do not occur in nature, can be frozen and kept indefinitely. Unlike animal cell cultures, the plant cells are totipotent and can be incorporated into breeding programs.

4.3 Freeze-Storage of Cell Cultures

A cell line to be maintained, has to be subcultured and transferred periodically and repeatedly over an extended period of time. It also requires much space and

manpower. Various methods have been tried by workers (SHERF, 1943; BUEL and WESTON, 1947; REISCHER, 1949; Caplin, 1959; MARTIN, 1964) to avoid periodical transfer of the cultures; however, they do not seem to be promising. Freeze preservation would be an optimistic approach to suppress cell division and to avoid the need for periodical subculturing.

4.4 Maintenance of Disease-Free Material

Pathogen-free stocks of rare plant materials could be frozen, revived and propagated when needed. This method would be ideal for the international exchange of such materials.

4.5 Cold Acclimation and Frost Resistance

Tissue cultures would provide a suitable material for the study of the mechanism of cold acclimation of plants at a cellular level and the effective temperature regimes for enhancing the hardiness of callus to cold (TUMANOV *et al.*, 1968; STEPONKUS and BANNIER, 1971). From the cultures subjected to super-low temperatures, it might be eventually possible to select "cold-resistant mutant cell lines" which could later be differentiated into "frost-resistant plants".

4.6 Retention of Morphogenetic Potential

Long-term tissue cultures are known to lose their ability to undergo morphogenesis (TORREY, 1967; MEYER-TEUTER and REINERT, 1973). By freeze storage, morphogenetic potential could be retained (BAJAJ, 1976) over an extended period of time.

4.7 Slow Metabolism and Ageing

At super-low temperatures there is a total arrest of growth and the cells are in a metabolic inactive state. This would prevent or virtually "stop" the ageing process.

References see page 778.

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