Cryopreservation of Plant Cells and Organs

Editor

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PREFACE

In the rapidly emerging domain of biotechnology, plant tissue culture, from a modest start, has now attained a unique and respectable status, more so in the last decade. Since plant cells or organs are amenable, in most cases, to in vitro culture resulting in the regeneration of entire plants, tissue culture techniques have been extensively used not only in the rapid clonal multiplication and eradication of systemic pathogens, notably viruses, but also in addressing problems related to physiology, genetics, developmental biology, and biochemistry. Recently, it was also recognized that by an interdisciplinary approach involving tissue culture and cryobiology, it should be feasible to preserve plant organs and cell lines for extended periods of time for the purpose of long-term preservation of germplasm of crop species or experimental material in a genetically unaltered state. Although this area of research (cryopreservation of plant cells, tissues, and organs) is still in its infancy as compared to the developments made with mammalian systems, remarkable progress has been made during the last 5 years. The purpose of this volume, therefore, is to provide the reader with an in-depth look at the subject matter area, analyze various critical components involved in the methodology, and present the progress made so far in the cryopreservation of various kinds of plant material.

Since plant cells, tissues, or organs form the basic experimental material in the cryopreservation study, the book opens with a chapter on plant cell culture in order for the reader to gain an overall appreciation of this field of research. In my opinion, a clear understanding of the various events a cell is subjected to during freezing and thawing is imperative before cryopreservation is attempted. The following three chapters provide basic information concerning the principles, nature, and mechanism of freezing injury to plant cells at various levels and attempt to suggest various ways and means of overcoming them for devising successful cryopreservation protocols. In subsequent chapters the role of cryoprotectants in the viable freezing of plant cells and the cryopreservation studies carried out with several types of plant material are examined in detail.

The authors of various chapters are internationally known scientists in the area of their expertise. I am extremely grateful to the contributors without whose cooperation and help this assignment could not have been completed. If the information presented in this book generates added interest and research activity among people considering cryopreservation of plant cells, tissues, and organs, the very purpose of the book, in my opinion, is served.

K. K. Kartha

THE EDITOR

Dr. K. K. Kartha is a Senior Research Officer at the Plant Biotechnology Institute of the National Research Council of Canada, Saskatoon. He obtained his M.Sc. (Agriculture) and Ph.D. degrees in India. His Doctorate in Mycology and Plant Pathology was obtained from the Indian Agricultural Research Institute, New Delhi, in 1969. From 1970 to 1972, he did his postdoctoral research at the Institut National de la Recherche Agronomique, Versailles, France, in the laboratories of Dr. M. T. Cousin and the late Professor Georges Morel. It was Professor Morel who introduced him to plant tissue culture research.

He joined on staff of the Plant Biotechnology Institute in 1973. He has published over 50 scientific papers in the area of plant pathology, plant tissue culture and cryopreservation. He has also contributed several chapters in various books. In 1981 he was a recipient of the George M. Darrow Award for excellence in research from the American Society for Horticultural Science for his work on the cryopreservation of strawberry meristems and mass propagation of plantlets. He was an invited symposium speaker at several international symposia including the Rockefeller Foundation Conference on Genetic Engineering for Crop Improvement held in New York in 1980.

He is a member of the Society for Cryobiology, Canadian Society of Plant Physiologists, Canadian Phytopathological Society, International Association for Plant Tissue Culture, and a life member of the Indian Phytopathological Society. Currently he is the elected National Correspondent for Canada for the International Association for Plant Tissue Culture.

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Chapter 1

PLANT CELL CULTURE*

Friedrich Constabel

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I. INTRODUCTION

Over 40 years ago White and Gautheret initiated plant cell culture as a method of experimental morphology.[1,2] Early objectives were to parallel animal cell culture and demonstrate theoretically indefinite longevity of meristems. Since then the cell culture method has permitted substantial advances in understanding growth and differentiation of plants. A cornerstone was the realization of totipotency of nonzygotic cells by Steward and Reinert.[3,4] More recently, plant cell culture has become a tool of geneticists and is considered ancillary to analyzing and manipulating the plant genome and to improving crops. Cryopreservation of cells and tissues is an integral part of this effort.

II. DEFINITION

The term *plant cell culture* refers to a variety of techniques which permit the growth and development, in vitro, of protoplasts, single cells, tissues, and organs derived from seed plants and ferns in a well-defined environment. As with animal cell culture, plant cell culture is not to suggest a suspension of uniformly single cells as can be obtained with microorganisms. The term is justified, however, when viewing the general objective to be attained by manipulating plant material at the level of cells.

III. METHOD

Plant cell culture requires the excision of a piece of tissue from a given plant and transfer of this tissue, the explant, to a nutrient medium formulated to induce and sustain cell multiplication and formation of new growth, callus. Step-by-step descriptions of procedures are presented in various laboratory manuals.[5,6] Isolation of callus from the explant and serial subculture in fresh media leads to the establishment of a callus strain or cell line. Employing liquid media and agitation will result in suspension cultures of smaller and smaller callus pieces, the formation of a cell suspension. Similarly, callus and cell suspensions can be obtained by starting cultures with cells freed from their walls, i.e., protoplasts.

The problem of not being able to create permanent, true cell suspensions of seed plants has often been attacked.[7] Sieves, wall-digesting enzymes, and a variety of hormones have been employed, but to no avail. The formation of a phragmoplast and primary wall between daughter cells appears to be an integral part of cell division and, so far, not accessible to biochemical or mutational inhibition. Callus, then, remains the predominant form of cell material open to in vitro culture.

IV. CALLUS

Coined for wound tissue, the term callus here refers to a more or less unorganized mass of parenchyma cells sometimes including sklerenchyma, i.e., tracheids. Callus originates from parenchyma cells of the explant by way of cell division. Thus, the size of the initial callus can be a function of the amount of parenchyma in the explant. Callus growth is sustained by the activity of meristematic cells which may remain diffuse or be grouped in islands and zones not unlike cambia. In time callus may attain a diameter of several centimeters. As a consequence, physiological gradients will establish themselves. This development would clearly contravene the purpose of plant cell culture, i.e., the study of growth and differentiation of cells outside gradients as characterize cells *in situ*. Subculturing calli is easily accomplished. The heterogeneity of this material, however, poses the problem of selecting those pieces as inocula for transfer which best suit subsequent experimentation. For example, selecting the fastest growing portions may result in fine-cell suspensions and

simultaneously in loss of morphogenetic capability. More than any other aspect of plant cell culture, growing callus can, therefore, truly be called an art.

V. NUTRIENT MEDIA

Growth of callus and cell suspensions is sustained by nutrient media. The most common media have been formulated by Murashige and Skoog for tobacco-pith tissue and by Gamborg et al. for soybean cell suspensions.[8,9] The merits of these two media have been discussed by Gamborg et al.[10] Besides mineral salts, carbohydrates, and vitamins which constitute such media, it is their fourth component, the hormones or growth regulators, which affects callus growth. While plant cell culture was thought to be primarily a method to detect and assay plant hormones and to analyze their molecular mode of action, it has been the response of plant cells to hormone treatment which has filled plant cell culture libraries. Only the detection of 6-furfurylaminopurine was related to growing tissues in vitro.[11] High-pressure liquid chromatographic and immunological analytical techniques have replaced bioassays today.[12]

In attempts to elucidate the reaction of cells to hormones, the attack is directed at the receptor site and at the reaction product. Libbenga et al., using tobacco cells and protoplasts, identified soluble in addition to insoluble, i.e., membrane-bound receptors for auxin.[13,14] These authors demonstrated binding of auxin to a receptor protein. The hormone-receptor protein complex formed specifically binds to certain nonhistone chromatin proteins called acceptors. This reaction, then, brings about an increase in template availability. The first response of hormone to follow is probably the synthesis of new mRNAs which are translated into new proteins, regulatory proteins which in turn derepress additional genes. For cytokinins there is similar strong evidence in favor of a translational control of growth.

The attack aimed at the reaction products to analyze the mode of action of hormones appears impeded by the difficulty of identifying such a product. Sung and Okimoto identified proteins which occur in carrot cell cultures upon elimination of auxins from the media and onset of embryogenesis.[15] Sussex described marker proteins which appear during a period of extensive growth of embryos.[16]

Media after Murashige and Skoog, and after Gamborg et al., have proven to be well-balanced nutrients and, therefore, have found world-wide acceptance. In this way the two media have greatly contributed to some standardization of callus and cell-suspension culture. Advances in methodology require, however, that new media are formulated to provide for growth of novel materials or growth in novel environmental conditions, i.e., for growth of anthers and microspores, immature embryos, for protoplasts, hybrid cells, mutants, and cells cultured at low density.[17-23] Most of these media are characterized by a numerical increase of components, carbohydrates, vitamins, C3 acids, complex substances, spent or conditioned media, or substances emanating from feeder layers of immobilized, X-rayed or actively growing cells.[24]

Callus and cell suspensions, even when green and exposed to light and good aeration, will not grow without an adequate supply of digestible carbohydrates. Attempts to increase the concentration of chlorophyll and differentiation of chloroplasts in cultured cells, thereby aiming at photoautotrophy and reduction of the nutrient media to mineral salts have been numerous. Adequate callus has been selected for green pigmentation with tobacco tissue over many subcuitures, using media with 3% sucrose, 2 μM NAA, and 6 to 14,000 lx, or with *Chenopodium rubrum*, using media with 3 to 0.5% glucose and 0.01 μM 2,4-dichlorophenoxyacetic acid and 4 to 8000 lx.[25,26] Today these cultures grow as fine, deeply green cell suspensions in the presence of only mineral salts, 1% CO_2, and light. This condition is thought to be most desirable for plant cells. One may expect, therefore, that more species will be brought into such an environment in the future. Photoautotrophy would also permit the closest approach of standardized plant cell culture.

VI. GROWTH

Growth by cell division is the result of one or a sequence of cell cycles. The control of cell cycles would appear to be a problem studied most suitably with synchronized cells cultured in vitro. Cell suspensions, indeed, have been amenable to synchronization, i.e., with an amplification factor which would allow biochemical analysis of phased nuclei and cells. Still, the perfection in synchronization as achieved with mammalian cells due to unique behavior of mitotic cells has not been paralleled with plant cells. Experimentation with hydroxyurea permitted 34% of cells to enter mitosis in synchrony.[27] A combination of FUdR (1 μg/mℓ) and uridine (0.5 μg/mℓ) induced 75% of cells to simultaneously enter mitosis.[28] Difficulties in synchronizing cultured plant cells are twofold: (1) lack of uniformity of starting material and (2) relative slowness in response of cells to inducing factors. Good results have been obtained with parenchyma cells of explants and with fine suspensions of rapidly growing cells of sycamore.[29,30] The problem involved in applying antimetabolites to induce synchrony is the necessity to subject the cells to several washes with fresh media, thereby removing conditioning factors required for rapid onset of growth. Constabel and Kurz employed nitrogen, and later, ethylene and CO_2 for temporary arrest of cells without disturbing media conditions.[31,32] Results obtained with synchronized material allowed calculation of the duration of individual phases of the cell cycle for various species. For example, the mean cycle time for sycamore cells was found to be 22.3 hr.[33] Synchronization also permitted demonstration that DNA replication in plant cells follows a pattern found earlier in mammalian cells, i.e., small intermediates of DNA were synthesized and then joined together, eventually yielding replication units which varied in length from 5 to 50 μm and in turn formed clusters of about 100 genes.[34]

Growth kinetics have been established for many callus and cell suspension cultures. They usually are the basis for studies directed towards cell development, specialization, and regenerative processes. Truly sigmoid growth curves resulted in a few instances only, i.e., when using most rapidly growing cell suspensions. Still, in most cultures a lag phase, a phase of accelerated ("logarithmic") and of decelerated and stationary growth can be identified. Of increasing interest are the growth kinetics of photoautotrophic cell cultures. Using material derived from *Chenopodium rubrum*, Hüsemann observed that during a 14-day growth period the increase in cell density and fresh weight followed a lag period of about 1 to 2 days, a 4-day period of exponential growth (60 to 80% increase in cell number in 2 days) with doubling times of about 55 hr, 4 days in which the rate of cell division declined to a 20% increase in cell numbers in 2 days, and stationary growth to day 14.[35] The time course of increase in chlorophyll of photoautotrophic cell cultures was linear except for an initial lag phase of about 4 days. Remarkably, these cells can be maintained in exponential growth without loss of photosynthetic activity. This behavior would indicate simultaneity of growth and differentiation.

VII. DIFFERENTIATION

Genetic, physiological, and morphological processes which bring about the specialization of cells are referred to as differentiation. Cells which have completed their development and have acquired a special function within the plant organism are differentiated cells.[36] The analysis of factors which control differentiation is often called morphogenesis. In the context of plant cell culture hormones, nutrients, light, and temperature, all have been identified as morphogenetic factors.

The focal point of interest in differentiation of plant cells grown in vitro is the observation that such cells are able to realize totipotency, i.e., to differentiate and grow into entire, functional plants theoretically identical to the original plants. The number and variety of

species which have successfully been made to develop from a cell or even protoplast to plants is remarkably great and still growing.[37-39] While immensely fertile as a concept and profitable as it permits industrial plant propagation, totipotency today is subject to sober scrutiny because of mounting evidence against its general applicability. First, cells grown in vitro may undergo genetic changes which often result in loss of totipotency. Secondly, a variety of materials will not respond with any differentiation and plant regeneration even under permissive conditions. Thirdly, plants regenerated from cells cultured in vitro do not necessarily perform as well as the original plants. Still, totipotency is fundamental to experimentation directed to better understanding and exploitation of differentiation, specifically the formation of plant products, of roots, shoots, and embryos.

A. Phytoproducts

Since their inception, cells cultured in vitro have been observed to synthesize and accumulate storage proteins, fats, carbohydrates, a variety of antibiotics, and secondary products. The latter have received most attention because they are the simplest products of morphogenetic processes, can be used as markers in somatic-cell genetics, and are industrial commodities. Typically, they are species specific and structurally occur in an almost infinite variety. Terpenoids, alkaloids, flavonoids, and glycosides are the most prominent groups of secondary metabolites. In plants, their formation is controlled by genomic, physiological, and morphological conditions. In *Sinapis alba*, for instance, cyanidin glycosides are formed as a response to photomorphogenetic factors in the lower epidermis of cotyledons and subepidermis of hypocotyls.[41] The challenge with plant cells cultured in vitro would be to devise genomic and physiological conditions, and to compensate for morphological conditions to permit product formation. Cloning cells selected for high yields and varying the nutritional and physical conditions of such cells would be the arsenal of tools to accomplish this.

Cells which undergo division may contain secondary metabolites.[42] In general, however, product formation occurs after cells have ceased to divide and entered what has been referred to as the idiophase. In praxi, this condition is attained when cells have exhausted nutrients of the media or upon transfer to media with reduced levels of hormones and nutrients. A number of media which promote product formation have been defined; the most widely accepted is the alkaloid production medium of Zenk et al.[43] In comparison to nutrient media mentioned earlier designed to stimulate growth, Zenk's medium features a low concentration of nitrate, phosphate, and auxin, and sucrose at a level of 5%. The carbohydrate is added not only as a source of energy, but also as material to be converted to secondary products. Light source and photoperiod as well as temperature, aeration, agitation, and pH, may be morphogenetic factors equal in importance as are media components.

Since publication of results by Zenk et al.,[43] it is generally agreed that, on the average, callus and cell suspensions derived from high-yielding plants are higher yielding than those from low-yielding plants. The observation of aberrant, low-yielding or nonproducing cultures can be explained by the heterogeneity of the explanted tissues as well as the mutations which may occur upon in vitro culture, specifically upon exposure to high concentrations of auxins.

Some secondary products are preferentially accumulated in cells with special structural features, i.e., in glands, ducts, laticifers. Callus and cell suspensions do not attain this stage of differentiation. It therefore appeared doubtful whether material cultured in vitro would accumulate respective products, i.e., mint oil terpenoids or latex alkaloids, for instance. More recently, however, menthone and menthol, components of mint oil, and codeine, a component of poppy latex, have been detected in cultured cells.[44,45] The biosynthetic capacity of plant cells appears not genetically linked to simultaneous structural differentiation.[46]

The significance of plant cell culture for fundamental research of secondary metabolism may be highlighted by showing inroads made by Hahlbrock et al.[48] into regulatory mech-

anisms. With cell cultures derived from parsley *(Petroselinum hortense)*, light was demonstrated to induce a staggered increase in the activity of phenylalanine-ammonia lyase and of chalcone synthase. The light-induced increases and subsequent decreases were found to occur with a high degree of coordination at both the enzyme and respective mRNA activity levels. Using chalcone synthase-specific cloned cDNA as a hybridization probe permitted demonstration that the changes in translatable mRNA activity were paralleled by corresponding changes in the amount of mRNA extractable from the cells. The results suggested that the observed light effect was a true induction phenomenon requiring *de novo* enzyme synthesis.

Phenylalanine-ammonia lyase activity has also been studied with tobacco-cell cultures.[49] A cell line resistant to *p*-fluorophenylalanine showed an enzyme activity which exceeded the nonresistant cell culture by a factor of 10. The resistant cell line did not respond to light, while cells of the sensitive culture showed a three- to fivefold induction of enzyme activity by light, analogous to the parsley cell culture.

B. Embryogenesis

The ability of callus cells to grow into roots and shoots or embryos which subsequently may develop into functional plants is the most perfect expression of totipotency. Spontaneous occurrences in nature are rare; cell material cultured in vitro has been observed to display embryogenesis comparatively often. While carrot cells appear to be the material of choice, embryo formation has been seen with over 80 species of over 33 families.[50]

Developmental stages are the same as found with respective zygotic embryos, i.e., in many dicotyledonous species, globular structures grow into heart-shaped, then torpedo-shaped embryos, clearly showing cotyledons, hypocotyl, and radicle. The presence of a suspensor is disputed; instead there is a connective tissue which links embryos with callus or explant.[51]

Apart from its practical application, interest in embryogenesis is focused on events which permit its control. While it is well established that embryogenesis is triggered by elimination of the external supply of auxins, the molecular basis of it is less clear. Two approaches have been made. For one, Sung and Okimoto have detected proteins which appear temporarily upon induction of embryogenesis through changes in the hormonal regime of the cultures.[15] The nature and function of these proteins are still under investigation. Also, through appropriate fractionation Fujimura and Komamine obtained synchronized embryos.[52] This material would permit biochemical analysis of factors which govern the pattern of development.

The occurrence of embryogenesis is thought to require the presence of competent cells in cultured material. Such a concept would help explain the frequency and failure of embryo formation in a variety of species and in a variety of tissues of one given species. The problem appears to be particularly acute in cereals where only specific sectors of the scutellum of immature embryos respond to permissive conditions.[53,54] It is this very observation which also most clearly invalidates the general applicability of the hypothesis of totipotency. Embryo formation, then, would depend on the preservation of a lineage of competent cells through callus formation and subculture; hormone elimination from the medium does not induce embryogenesis, but merely triggers competent cells to express an otherwise latent developmental pattern.

The gradual loss of ability to produce embryos in established cell cultures is a common observation. This phenomenon would likewise be explained by termination of the lineage of competent cells. Genetic changes of respective cultures have been described and may well account for changes in embryogenic activity.[55]

Embryo formation from microspores has attracted special attention, primarily for practical reasons, the growth of haploid plants.[56] Embryos from microspores of *Brassica napus* germinated to form plantlets which in turn produced large numbers of secondary embryos.[57]

Sections through stems undergoing secondary embryogenesis showed that the embryos arose from single cells of the epidermis. Some of the resulting plantlets were demonstrated to be haploid.

The culture of embryos was extended to immature seeds of a variety of plants and was of considerable consequence in efforts to rescue embryos from abortion in sexual hybrids, critical for plant-breeding programs. Reports of the successful culture of embryos continue to appear and include clover, alfalfa, triticale, *Ilex*, *Viburnum*, coconut, pea, peanut, barley x rye, maize x sorghum. In vitro fertilization techniques are beginning to receive attention. A few interspecific and intergeneric hybrids have been made by first accomplishing fertilization in vitro, then culturing ovaries to maturity.[18,58]

VIII. CYTOGENETICS

Plant regeneration from cells will find its most significant application when in tandem with genetic cell modification. The result will be genetically modified plants and, more desirable, improved crops. Plant cells cultured in vitro have long been observed to change spontaneously; more recently, and particularly with the availability of protoplasts, they can be subjected to a series of manipulations. The growth of somatic hybrid plants would epitomize today's state-of-the-art.[59]

A. Variation

Spontaneous changes in cultured cells may affect the texture and pigmentation of a callus, nutritional requirements, loss of ability to regenerate plants, and ability to synthesize and accumulate secondary metabolites. Spontaneous changes are a reflection of the variability of cells grown in culture. They occur more frequently with material exposed to high doses of hormones, in particular 2,4-dichlorophenoxyacetic acid, used to stimulate and sustain growth of callus and subcultures. Karyological investigations revealed that callus often displayed chromosomal variation, i.e., polyploidy and aneuploidy. Ashmore and Gould demonstrated remarkable chromosomal variation, including translocations and inversions upon explantation, callus formation and subculture with *Crepis capillaris*.[60] Earlier, Bayliss had documented, with cell suspensions of carrot the kinetics of di-, poly-, and aneuploid cell populations in long periods of culture, preferred growth of one kind of population under one given culture condition.[61] Bayliss also summarized those cell cultures which, over years, appear not to have deviated from diploidy.

Genetic changes which accompany callus formation and subculture cannot be avoided, only mitigated, i.e., by nutrient media with low levels of hormones. The resulting variation in phenotype may be exploited for the selection of cells resistant to a variety of antibiotics, pathogens, and for the selection of cells with improved product formation.[62,63]

Filner reported slow adaptive changes in urease levels in tobacco cells grown on urea instead of nitrate as the sole source of nitrogen.[64] The urease activity of the cells increased over several months to levels which permitted cell growth as if in the presence of nitrate. Upon return of the cells to media with nitrate, the urease activity decreased. However, when the cells were again transferred to urea, urease activity quickly rose to levels as found formerly and before return to nitrate. Filner proposed gene amplification as a possible molecular basis for this variant phenotype. The induction of high urease activity by urea in a single step in rare cells and the induction of higher urease activity in a continuum of small increments in all cells has not been ruled out. However, the occurrence of a mutational event as the basis for this altered phenotype appears to be excluded by evidence such as (1) the high frequency of occurrence and (2) the short-term stability (two transfers) of the high urease phenotype in the absence of the selective pressure of urea.

B. Habituation

The induction of callus formation with explants and maintenance of subcultures of such callus generally require the presence of hormones in the nutrient medium. Sometimes, however, subcultures lose their requirement for hormones and can be maintained without external supply of hormones; material cultured has acquired the ability to produce hormones in amounts sufficient to promote growth. The phenomenon was first observed by Gautheret and referred to as habituation. Binns and Meins discovered habituation for auxin and for cytokinin.[65] Upon analysis, habituation was described as stable at the cellular level because in cell clones this trait was inherited by the progeny. Habituation also proved to be reversible, because callus from regenerated plants of habituated cell clones reverted to hormone requirement. Therefore, habituation was demonstrated to be epigenetic in nature.

Results would highlight the need for rigorous experimental analysis of variation in cell cultures, including cloning, subcloning, and plant regeneration before an opinion on the genetic or epigenetic nature of such variation is given.

C. Mutation

Mutant cell lines are desirable for two reasons: they may show improved yields of phytoproducts, alkaloids for example, and they may provide a marker system to be used in subsequent genetic and biochemical experimentation. Mutagenic agents, physical and chemical, and their effects on plant cells cultured in vitro have been well defined. It may be added that dose-response curves differ for each cell line. The problem with mutant cell lines is their leakiness. It results from defects being associated with recessive genes, the presence of multiallelic genes, or contamination of the mutant by wild-type cells. The mutagenic system would be improved by aiming at mutations or dominant genes of traits coded for by only one pair of allels and by stringent selection procedures.

Haploid cells grown by way of anther and microspore culture showed experimentally induced mutation. Subsequent diploidization can be achieved by colchicine treatment and cell fusion. As a result, mutations have been obtained in a homozygous state in doubled amphidiploid cell lines. Regeneration led to respective mutant plants, with *Nicotiana tabacum*, for instance.[66]

D. Cell Fusion and Hybridization

The establishment of techniques to isolate and culture plant cells without walls, protoplasts, opened the way to cell fusion. The discovery of polyethylene glycol and a few other chemicals as powerful fusogenic agents has made the generation of somatic-cell hybrids reality.[67] Several cell hybrids have been grown to plants showing more or less intermediate character.[59] Critical in all cases was the application of successful hybrid selection systems. Cocking lists those methods which have proven useful in selecting hybrid callus and plants.[68]

1. Use of selective media for the selective growth of somatic hybrids (*Nicotiana glauca + N. langsdorffii*).[69]
2. Use of light-sensitive, chlorophyll-deficient mutants for complementation selection (*N. tabacum + N. tabacum*).[70]
3. Use of complementation selection, coupled with differential media growth, by fusing wild type and albino protoplasts (*Petunia parodii + P. hybrida*).[71]
4. Use of biochemical mutants for complementation selection of somatic hybrids (*N. tabacum + N. knightiana*).[72]
5. Use of two nonallelic albino mutants for complementation selection of somatic hybrids (*Datura innoxia + D. innoxia*).[73]
6. Heterokaryon selection by mechanical isolation (*Glycine max + N. glauca*).[74]

Once generated, hybrid cells may be employed in answering a number of questions on

speciation and incompatibility of species, on chromosome segregation and gene mapping, and on the inheritance of cytoplasmic traits.

Somatic cell hybridization has been used for the transfer of male sterility. Through cell fusion, cytoplasm was transferred from irradiated *N. tabacum* to *N. plumbaginifolia* cells with streptomycin resistance as the marker. By selection for streptomycin resistance, cytoplasmic male sterility, a mitochondrial trait, could be transferred.[75]

E. Gene Transfer

Several avenues are being explored to arrive at gene transfer. One way has been found in generating fusion products, using as partners protoplasts with nuclei in metaphase. Here, chromosome segregation is achieved not during long-term hybrid cell culture, but with the initial step, i.e., fusion.[76] Individual DNA segments may be packaged with membranes, i.e., enclosed by lipid vesicles and made to fuse with host cells.[77] Finally, and with remarkable success, plasmids may be used as vectors for gene transfer.[78]

Ti-plasmids, carried by *Agrobacterium tumefaciens*, have been shown to be responsible for crown gall formation in plants. Ti-plasmids are natural gene vectors with which *Agrobacterium* achieve the transfer and stable maintenance of a defined DNA segment (called T region) into the nucleus of transformed plant cells. Using site-specific mutagenesis, it was possible to introduce mutations in different parts of the T region. The transcription of the T-DNA in wild-type and mutant crown galls was compared and it was found that the induction of specific developmental patterns could be correlated with the presence or absence of specific T-DNA transcripts. Double mutants were obtained in which the expression of all the genes for tumor formation was abolished. Tobacco, potato, and petunia plant cells harboring the inactivated T-DNAs were shown to regenerate normal, fertile plants which transmit the T-DNA segment as a single Mendelian locus.[79]

IX. CONCLUSION

Cell cultures have proven to be an extremely useful tool, because (1) they reduce biochemical processes that occur in whole plants to cells or groups of cells which can be kept under strictly controlled environmental conditions, (2) they can be obtained as haploids, (3) they can be genetically modified through a variety of mutations and hybridization, and (4) they can be made to regenerate plants and thus be employed in plant propagation and plant breeding. Cells in vitro, however, are also prone to spontaneous changes. While this variability may be desirable in some instances, generally it is undesirable and should be prevented as far as possible. Plant cell culture, therefore, has to be supplemented by cryogenic methodology.

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