Cloning Agricultural Plants Via In Vitro Techniques

Editor

B. V. Conger, Ph.D.

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PREFACE

Plant cell and tissue culture generated much excitement during the 1970s concerning the potential application of the technology for improving important agricultural crop plants. This originates from the demonstration of cellular totipotency, or the ability to regenerate whole plants from single cells, and the successful creation of hybrids by somatic cell fusion in some species. There are several areas of in vitro culture which have potential practical application. However, currently the most practical application is for cloning or mass propagation of selected genotypes. This is evidenced by the large number of commercial firms engaged in propagating a variety of plants through tissue culture.

The purpose of this book is to provide a reference guide on principles and practices of cloning agricultural plants via in vitro techniques for scientists, students, commercial propagators, and other individuals who are interested in plant cell and tissue culture and especially its application for cloning. The chapters are categorized according to major classes of crop plants and each were written by a different author who has expertise for that particular crop class. Different approaches were taken by different authors and no attempt was made to achieve a uniform style or approach nor to completely avoid duplication. Basic principles as well as actual practices for specific crop species are presented. The ratio of principles to practices varies with each chapter depending on the approach taken by the author. Each chapter is followed by an extensive list of references which the reader may consult for additional information. There is, of course, a problem in such a popular and rapidly expanding field in including all current references, since many new publications appear each week. However, the inclusion of new material must cease at some point. The chapters should include most of the pertinent publications up to the end of 1979 when the manuscripts were due.

I wish to express my sincere appreciation to the other authors of this book for their excellent contributions. Appreciation is also extended to the Department of Plant and Soil Science and the Agricultural Experiment Station of the University of Tennessee for providing funds, time, and services essential for completion of this treatise. Final editing and completion of Chapters 1 and 5 were also partially supported by the Competitive Research Grants Office of the U.S. Department of Agriculture under Agreement No. 5901-0410-9-0331-0.

B. V. Conger
January 1980

THE EDITOR

Bob V. Conger, Ph.D., is a Professor of Plant and Soil Science at the University of Tennessee, Knoxville. He received his B.S. in Agronomy from Colorado State University, Fort Collins in 1963 and his Ph.D. in Genetics from Washington State University, Pullman in 1967. He was supported in his doctoral research by a National Aeronautics and Space Administration Predoctoral Traineeship.

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Dr. Conger has presented more than 20 papers at National and International Meetings and published more than 50 scientific papers. He has served as a member of a Food and Agriculture Organization of the United Nations/International Atomic Energy Panel on Improvement of Mutation Breeding Techniques and is currently an Associate Editor of *Environmental and Experimental Botany*. His current research interests are in the areas of plant cell, tissue culture, and breeding forage grasses.

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Chapter 1

INTRODUCTION

B. V. Conger

Not only is man absolutely dependent on plants for food, but plants are also a major source of clothing, fuel, drugs, and construction materials. Furthermore, as ornamentals they are both useful and aesthetically pleasing.[1] Cultivation and attempts to improve, maintain, and propagate useful and desirable plant genotypes predates recorded history. Also, it is not known when the first attempts were made to vegetatively propagate plants.

Plant propagation through tissue culture may have been considered as early as 1902 when Haberlandt[2] first attempted to regenerate plants from single cells. Although this pioneering work was unsuccessful, it inspired others to attempt in vitro culture of plant tissues. Very little progress was made during the 30 years following Haberlandt's paper; however, in 1934, White[3] established an actively growing clone of tomato roots. In the late 1930s, the first prolonged culture of unorganized plant tissue was reported independently in carrot by Gautheret[4] and Nobécourt,[5] and in tobacco by White.[6]

Experiments by Skoog and Miller[7] in 1957 showed that different auxin to cytokinin ratios influenced the type of growth and/or morphogenesis in tobacco. Organization and development of complete plants from a cultured mass of carrot cells was demonstrated by Steward et al.[8] in 1958. In 1962, Murashige and Skoog[9] published a defined medium for tobacco culture which has probably been cited more than any other for culture of a wide range of plant species, including both dicots and monocots. Using a defined medium, Vasil and Hildebrandt[10] obtained differentiation of completely organized tobacco plants from single cells in 1965. In 1969, Nitsch and Nitsch[11] published a method to grow hundreds of haploid tobacco plants from pollen grains.

Based on demonstrations in the early 1970s that protoplasts isolated from mesophyll cells could be induced to generate into entire plants[12] and that protoplasts could be stimulated to fuse under defined experimental conditions,[13] Carlson et al.[14] created the first parasexual hybrid by fusing protoplasts from two tobacco species in 1972. The event, perhaps more than any other, was one of the most significant from the standpoint of expanding interest, publicity, extramural funding (both public and private), and enticing researchers who had little or no previous interest to conduct research in plant cell and tissue culture.

The above represents only a few of the significant developments in plant tissue culture. For a more thorough historical background the reader is referred to the introductory chapter in the book edited by H. E. Street.[15]

There are several potential practical applications of plant cell and tissue culture in agriculture. These include:

Induction of haploid plants from anther and pollen culture — These are of great interest in plant breeding since recessive mutations induced in them can be identified immediately and doubling the chromosomes leads directly to homozygous individuals.

Protoplast culture — The primary interest here deals with the possibility of modifying the plant's genome by uptake of exogenous DNA and creating hybrids between sexually incompatible species through protoplast fusion. Although there have now been several reports of successful interspecific and intergeneric hybrids produced by the protoplast fusion (including some between sexually incompatible species)[16,17] the number of species in which plants can be regenerated from protoplasts is very small.

There have been no published reports of plant regeneration from any of the important cereal and legume crops, fruit crops, or trees.

Induction and selection of mutants — The identification of mutants in haploid cultures was mentioned above; however, this topic is also of interest in culture of somatic tissue. For example, induction and isolation of mutants from sugarcane tissue cultures which are resistant to various diseases have been especially successful.[18]

Clonal propagation or rapid multiplication of specific genotypes — The use of in vitro techniques for asexual propagation is the most advanced area of plant tissue culture. Thus, it is also the area that currently has the most practical application.[19,20,29] According to Murashige,[20] by 1978 there were at least 100 facilities engaged in commercially propagating a variety of plants through tissue culture and nearly all of them arose within the previous 5 years. Also, several new establishments continue to arise each year. The differences between tissue culture and traditional methods for cloning involve the use of smaller propagules, the provision of an aseptic and artificial environment, and substantially faster plant multiplication.[21]

Currently, cloning by either conventional or in vitro techniques is especially valuable for propagation of heterozygous, sexually incompatible, and sterile genotypes. These characteristics occur in many of our ornamental, vegetable, and fruit crops. However, to eventually exploit other novel techniques of plant tissue culture, e.g., parasexual hybridization, haploid culture, etc., in the improvement of important agronomic crops and trees the ability to rapidly clone these species in large numbers may also be necessary.

Hussey[22] and de Fossard[23] have listed a number of reasons for efficient and reliable vegetative propagation which may utilize plant tissue culture techniques. These include:

1. Rapid multiplication of new hybrid cultivars, which arise as single plants, for testing and eventual commercial production
2. Elimination of viruses from infected stocks
3. Vegetative propagation of difficult to propagate species
4. Year around propagation of clones
5. Propagation of genetically uniform parent plants in large numbers for large-scale hybrid seed production

Rapid asexual multiplication can be obtained through (1) enhanced precocious axillary shoot formation, (2) production of adventitious shoots either directly on organ explants or in callus, and (3) somatic cell embryogenesis.[21,22] Production of shoots in (1) or (2) above is followed by rooting of individual shoots to produce complete plants. The production of plantlets from callus has the potential for very rapid multiplication of large numbers. However, there are serious problems with some species in difficulty or even complete failure to regenerate plants from callus or liquid cultures and high rates of genetic and/or cytogenetic altered plants. These problems are discussed in later chapters of this book.

Asexual embryogenesis is potentially the most rapid method of cloning plants in vitro.[21] The embryos can arise directly from the explant or from an intermediary callus. However, this method, as presently known, is not a desired way to propagate plants. Its use requires synchronous development and the embryos must be protected with a coating for automated sowing in the soil, prolonged storage, and easy transport.[21] Also, there is a problem with phenotypically altered plants which may result from genetic or cytogenetic changes.

Murashige[21,24] lists a sequence of stages or steps which plant propagation through

tissue culture must proceed starting with the explant through establishment of the plants in the soil. These steps have been either directly or indirectly discussed in other chapters of this book, e.g., the chapter on ornamental species discusses the various requirements for each of these stages.

In vitro techniques for cloning or mass propagation are defintely more advanced and currently have greater application in some plant species than in others. Orchids were the first plants to be propagated by tissue culture. Also, the elimination of somaic virus by shoot apex culture was described in the orchid *Cymbidium* by Morel.[25] This technique has subsequently been employed to eliminate a multitude of viruses from a variety of other plant species. Morel[25] estimated that as many as 4 million *Cymbidium* could be produced from a single explant in 1 year.

Asparagus is another crop which has been successfully propagated by in vitro methods. It has been estimated that 300,000 transplantable plants may be produced from a single shoot apex culture in a year.[26] In actual practice, however, 70,000 plants can be produced by one person working 200 days 1 year.[27] This level of production depends on an adequate supply of aseptic stock plants and the culture of 500 bud segments by one person in 1 day.

In some agricultural plants, e.g., most agronomic crops, in vitro techniques are being used very little, if at all, for propagation.[28] Furthermore, these techniques may never find application for some of these crops. The current status and potential uses, however, have been examined. Even though major advances have been made in plant tissue culture, including in vitro propagation, of many important agricultural crop plants, there are several major problems and limitations which must be overcome before these techniques can become a routine method of plant propagation in a wider range of species. Some of these include failure to regenerate plants from callus and cell cultures and, as mentioned above, maintenance of genetic and cytogenetic stability. Other problems include internal contamination by microorganisms in explants of certain plants which is difficult to eliminate and thus hinder or even preclude culture.

The purpose of this book is to review the principles and practices for cloning various classes of agricultural crop plants via in vitro techniques in terms of currently available knowledge. The requirements for tissue culture propagation are discussed in both a general sense and in some cases for specific crops in the various chapters. The associated problems as well as potentials for the future are also discussed. Although the primary emphasis is on the use of in vitro methods for cloning, other principles, practices, and potentials of plant tissue culture receive various degrees of mention in the other chapters. Also, each chapter is followed by an extensive list of references on plant cell and tissue culture for that particular class of agricultural plants.

The chapters are categorized according to various classes of economically important agricultural plants and are entitled, Ornamental Species, Vegetable Crops, Fruit Crops, Agronomic Crops, and Trees. Since each chapter was written by a different author different approaches were taken. Hopefully, the book will provide the reader with a starting point for in vitro culture of the plant or crop species in which he or she is interested and from which modifications may be made to improve or optimize culture conditions and technique. Since not all chapters may contain all information concerning general principles or procedures, e.g., stages of in vitro culture or media formulations, the reader may consult the index to find this information from another chapter(s). If more detail for a specific procedure or plant species is needed the reader may consult the original references.

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Chapter 2

ORNAMENTAL SPECIES

Karen W. Hughes

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I. INTRODUCTION

Plant cell and tissue culture techniques have, in recent years, developed into very powerful tools for propagation of ornamental species. The technology had its beginnings with Haberlandt's speculations regarding cell totipotency at the turn of the 20th century.[1] Haberlandt suggested that techniques for isolating and culturing plant tissues should be developed and postulated that if the environment and nutrition of cultured cells were manipulated, those cells would recapitulate the developmental sequences of normal plant growth. Plant tissues were first successfully cultured by White in 1934.[2] By 1939, White, Nobécourt, and Gautheret had reported the first successful callus cultures of carrot and tobacco.[3-5] In 1957, a key paper by Skoog and Miller was published in which they proposed that quantitative interactions between auxins and cytokinins determined the type of growth and/or morphogenic event that would ensue.[6] Their studies with *Nicotiana tabacum* indicated that high auxin to cytokinin ratios induced rooting while the reverse induced shoot morphogenesis. Unfortunately, this pattern of response is not universal. While manipulations of auxin to cytokinin ratios have been successful in obtaining morphogenesis in many taxa, it is now clear that many other factors affect the ability of cells in culture to differentiate into roots, shoots, or embryos.

A major stimulus for application of plant tissue culture techniques to the propagation of ornamental species may be attributed to the early work by Morel on the propagation of orchids in culture[7] and to the development and widespread use of a new medium with high concentrations of mineral salts by Murashige and Skoog.[8] Following success with rapid in vitro propagation of orchids, plant cell and tissue culture techniques were applied to other species with varying degrees of success. Herbaceous ornamentals have been relatively easy to propagate via tissue culture, but success has been elusive with some taxa including woody perennial species and with members of specific families such as the Favaceae.

II. PROPAGATION OF ORNAMENTAL SPECIES

The in vitro multiplication of ornamental plants has several advantages over conventional methods of plant propagation.

1. The number of genetically identical plants recovered from a single stock plant is greatly increased. Through tissue culture techniques, a single stock plant may produce thousands or even millions of plants depending on the capability of the culture system.[9]
2. Disease free plants may be obtained. Plants which have been propagated by tissue culture techniques are free of superficial bacteria and fungi; however, internal pathogens (viruses and viroids) and some endosymbionts may be propagated with the plant tissue. Consequently, in some families, particularly the Orchidaceae, viruses are now widespread.[10] Virus-free plants may be obtained through meristem culture (see Section IV. B).
3. Stocks may be maintained in vitro where greenhouse space for maintenance of plants is at a premium.
4. Tissue culture techniques may be used to obtain hybrids from incompatible species through either embryo or ovule culture.
5. In a few ornamental species, haploid plants have been obtained through anther culture. Haploid plants have some advantages over diploid material when mutagenesis is used in that recovery of recessive mutations is enhanced. Further, doubled haploids are homozygous and thus pure-breeding.

Species of Orchidaceae are among the most extensively studied and propagated taxa. Tissue culture techniques have been used for rapid multiplication, for the production of virus-free orchids through meristem culture, and for germinating seeds. Several excellent reviews concerning the application of tissue culture technology to orchids are available and details will not be repeated here.[9,11-13] For reference purposes, genera of orchids which have been propagated through tissue culture are listed in Table 1.

Ornamental species from more than forty different families, excluding the Orchidaceae, have been propagated using tissue culture techniques. In some taxa, propagation has been applied on a commercial scale. In other taxa, propagation has been shown to be possible although not necessarily feasible from a commercial standpoint. Recent reviews by Murashige[9,154] on the propagation of higher plants and by Holdgate[155] on the commercial aspects of propagation are available. Fern species which show demonstrated potential for propagation through tissue culture are listed in Table 2. Flowering plant species, other than orchids, are listed in Table 3.*

III. PROPAGATION STAGES

Murashige has separated the steps in clonal multiplication into three stages: Stage I — establishment of an aseptic culture; Stage II — multiplication; and Stage III — rooting and preparation of the propagule for transfer to soil.[9]

A. Stage I

The first step in any plant cell or tissue culture system is to obtain a suitable explant. Almost any plant tissue or organ may be used for an explant depending on the purpose of the culture system and the species involved (Tables 2, 3, and Section IV. B). Surface contaminants, e.g., bacteria and fungi, must be removed prior to culturing. Disinfection may involve combinations of the following procedures:

1. The explant is washed in running tap water for 1 to 2 hr. This procedure sharply reduces the level of surface contaminants and, at least in the Gesneriaceae, does not seem to affect the viability of the tissues.[385] If the explant is especially pubescent, a prewash with detergent may help to wet the surface. Extended washing may break up colonies of surface bacteria and fungi making them more accessible to sterilizing agents. It may also reduce the population size of the surface contaminants.
2. Plant tissues are immersed in an antiseptic solution to kill remaining surface contaminants. A dilution of commercial laundry bleach (5 to 6% sodium hypochlorite) in sterile water is an effective antiseptic although the dilution level and time of exposure must be determined for each type of explant. A few drops of a detergent such as Tween 20 (polyoxyethylene sorbitan monolaurate) may be added to break the surface tension of the tissues. Tween 20 may be autoclaved in water. A 70% solution of ethanol may be used for sterilization; however, there is some evidence that ethanol may seriously affect plant growth response.
3. Following sterilization procedures, the explant is rinsed several times in sterile distilled water and all damaged tissue removed. The explant is subdivided where necessary and transferred to nutrient medium. [Tissues that brown rapidly may be dipped in a solution of critic and abscoric acid (100 to 150 mg l^{-1} each) to retard oxidation.[154]]

* Table 3 will be found at the end of the text.

Table 1
ORCHID GENERA IN WHICH TISSUE CULTURE TECHNIQUES HAVE BEEN SUCCESSFUL

Genus	Ref.
Acianthus	14
Aerides	15, 16
Aranda	17—22
Aranthera	22
Arundina	23
Ascofinetia	24
Bletilla	25
Brassavola	15, 16
Brassocattleya	26
Broughtonia	15, 16
Caladenia	14
Calanthe	27, 28
Calochilus	14
Calopogon	29
Cattleya	15, 16, 26, 28, 30—59
Cymbidium	7, 26, 43, 44, 49, 60—85
Cypripedium	29, 74, 86, 87
Dendrobium	15, 16, 28, 44, 50, 88—99
Dipodium	14
Diuria	14
Doritis	15, 16
Encyclia [*Epidendrum*]	100, 101
Epidendrum	15, 16, 102—107
Eriochilus	14
Eulophidium	26
Glossodia	14
Goodyera	29
Habenaria	29
Haemaria	108
Laeliocattleya	26, 102, 103, 109, 110
Liparis	29
Listera	29
Lycaste	44
Microtis	14
Miltonia	28, 43, 44
Neostylis	18
Neottia	111
Odontoglossum	44
Odontonia	27
Oncidium	15, 16, 27
Ophrys	27, 112
Paphiopedilum [*Cypripedium*]	26, 28, 106, 113—117
Phaius	44
Phalaenopsis	15, 26, 28, 32, 50, 113, 118—136
Pogonia	29
Potinara	26
Pterostylis	14
Renanthera	15
Rhynchostylis	137, 138
Schomburgkia	48
Sophrolaeliocattleya	26, 113
Spathoglottis	139, 140
Spiranthes	29
Thelymitra	14
Vanda	15, 17, 55, 138, 141—151

Table 1 (continued)
ORCHID GENERA IN WHICH TISSUE CULTURE TECHNIQUES HAVE BEEN SUCCESSFUL

Genus	Ref.
Vascostylis	24
Vanilla	86, 152
Vuylstekeara	27
Zeuxine	153
Zygopetalum	27

Table 2
FERN GENERA IN WHICH TISSUE CULTURE TECHNIQUES HAVE BEEN SUCCESSFUL[a]

Genus and species	Explant source	Morphogenic response	Ref.
Adiantaceae			
Adiantum cuneatum Langsd.	Rhizome tip	Adventitious shoots	9
Adiantum tenerum Swartz	Homogenized gametophytic tissues	Sporophytic plants	157
Blechnaceae			
Woodwardia fimbriata Grant c.f.	Rhizome tip	Adventitious shoots	9, 158
Cyatheaceae			
Alsophila australis R. Br.	Rhizome tip	Adventitious shoots	9, 158
Davalliaceae			
Davallia bullata Wall.	Homogenized plants from culture	Sporophytic plants	159
Nephrolepsis cordifolia (L.) Presl.	Runner tips	Sporophytic plants	160
Nephrolepis exaltata (L.) Schott var. *bostoniensis*	Runner tips	Adventitious shoots	9, 158, 161, 162
Nephrolepis falcata f. *furcans* Proctor	Runner tip	Adventitious shoots	163
Dennstaedtiaceae			
Microlepia strigosa (Thunb.) K. Presl.	Rhizome tip	Adventitious shoots	9, 158
Dyopteridoidea			
Cyrtomium falcatum Presl.	Homogenized gametophyte tissues	Sporophyte plants	157
Platycerioideae			
Platycerium bifurcatum C. Chr.	Homogenized gametophytic tissues	Sporophytic plants	157, 159
Platycerium stemaria Beauvois	Shoot tips	Adventitious shoots	164
Pteridoideae			
Pteris argyraea T. Moore	Rhizome tip	Adventitious shoots	9
Pteris cretica L.	Leaf stem	Gametophytes, sporophytes	165
Pteris ensiformis Burm.	Homogenized gametophyte tissues	Sporophytic plants	157
Pteris vittata L.	Rhizome callus	Sporophytes, gametophytes	166
Thelypteridaceae			
Cyclosorus dentatus (Forsk) Ching	Root apex explants	Gametophytes, sporophytes	167

[a] Assignment of genera to families follows that of Crabbe, Jermy, and Mickel.[156]

Some plant tissues have internal contaminants that can not be removed by standard techniques.[386,387] The addition of benomyl or benolate at 10 mg l^{-1} to the medium may prove effective in reducing fungal contamination; however, benomyl can affect subsequent plan development adversely.[388,389] Antibiotics may be added to inhibit bacterial growth, but antibiotics are often harmful to plant cells, particularly those which affect the ribosomes. Since antibiotics are heat labile, they must be filter sterilized.

B. Stage II

Several alternate procedures have been used for the clonal multiplication of plants including the so called "meristem culture", development of axillary and terminal buds, induction of adventitious shoots, and somatic embryogenesis.

1. Development of Axillary and Terminal Buds

Axillary and terminal buds may be induced to develop in vitro. A single bud may produce only a single shoot, or depending on the species and the medium, may produce multiple shoots. Occasionally, callus formation and the development of shoots from meristematic areas in the callus occur. If shoots formed from the buds, in turn, develop buds along their axis, the procedure may be continued indefinitely. Clonal multiplication by this procedure is limited to the number of buds available; however, the procedure may be applied to some woody taxa where adventitious bud development or somatic embryogenesis has not been successful.

Propagation through "meristem culture" is a special class of the above, but with much wider application. The true apical meristem is restricted to a very small area of actively dividing cells, devoid of leaf primordia, at the very tip of the shoot. Culture of the true apical meristem is rarely successful (see Section IV.B) and in practice, a larger shoot is usually excised and subcultured. The term "meristem culture" has been erroneously used to describe shoot tip culture.[9,10]

2. Adventitious Shoot Development

In many species, plant organs, e.g., roots, shoots, or bulbs may be induced to form on tissues which normally do not produce these organs. Such adventitious organogenesis has more potential than the induction of axillary buds for mass clonal propagation of plants. A single leaf, for example, may produce thousands of buds or shoots, each genetically identical to the explant. Numerous taxa are multiplied by this procedure (Tables 2 and 3).

3. Somatic Embryogenesis

The greatest potential for clonal multiplication is through somatic embryogenesis where, technically, a single isolated cell can produce first an embryo, then a complete plant. Somatic embryogenesis has been demonstrated in several highly morphogenic species to date including carrot, *Nigella, Antirrhinum,* and *Petunia*. Somatic embryogenesis may occur in suspension cultures or occasionally in callus. In the examples of somatic embryogenesis known to date, induction of embryogenesis requires exposure to an auxin, often 2,4-D, followed by a reduction in auxin levels. Embryo induction also required a source of reduced nitrogen.[390] Somatic embryogenesis has recently been reviewed by Kohlenbach,[390] Raghavan,[391] and Narayanaswamy.[392]

C. Stage III

Shoots induced in culture may spontaneously produce roots, but more often it is necessary to transfer shoots to another medium to induce rooting. In some taxa such as *Saintpaulia* and *Episcia,* shoots will root when transferred directly to the soil.[316,321]