EXPERIMENTS IN Plant Tissue Culture

SECOND EDITION

JOHN H. DODDS & LORIN W. ROBERTS

The second edition of *Experiments in Plant Tissue Culture* makes available much new information that has resulted from recent advances in the applications of plant tissue culture techniques to agriculture and industry. This laboratory text features an enlarged section on terminology and definitions as well as additional information on equipment and facilities. Also included is a nonexperimental section on two special topics: virus eradication and plant tumors and genetic engineering.

This comprehensive text takes the reader through a graded series of experimental protocols and also provides an introductory review of each topic. After an introductory historical background, there are discussions of a plant tissue culture laboratory, aseptic techniques, and nutritional components of media. Subsequent chapters are devoted to callus induction, organ formation, xylem cell differentiation, root cultures, cell suspensions, micropropagation, somatic embryogenesis, isolation and fusion of protoplasts, haploid cultures, storage of plant genetic resources, secondary metabolite production, and quantitation of procedures. A glossary of terms, a table of media formulations, and a list of commercial sources of supplies are also included.

The work offers all of the basic experimental methods for the major research areas of plant tissue culture, and it will be invaluable to undergraduates and research investigators in the plant sciences. It will also be useful to researchers in related areas such as forestry, agronomy, and horticuiture, and to those in commercial houses and pharmaceutical companies interested in acquiring the laboratory techniques.

Experiments in Plant Tissue Culture

Second Edition

This volume is dedicated to Professor Gautheret for his outstanding contributions to research and teaching of plant cell and tissue culture.

EXPERIMENTS IN
Plant Tissue Culture

Second Edition

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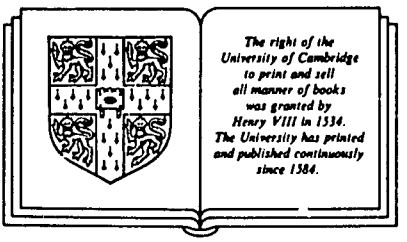

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CONTENTS

TABLES

FOREWORD

The idea of experimenting with the tissues and organs of plants in isolation under controlled laboratory conditions arose during the latter part of the nineteenth century, finding its focus in the work of the great German plant physiologist Haberlandt some 80 years ago. Haberlandt's vision was of achieving continued cell division in explanted tissues on nutrient media – that is, of establishing true potentially perpetual, tissue cultures. In this, he was himself unsuccessful, and some 35 years were to elapse before the goal was attained – as it could be only after the discovery of the auxins. Gautheret, Nobécourt, and White were the pioneers in this second phase. The research they set in train was at first mainly concerned with establishing the conditions in which cell division and growth would take place in explants, and in exploring the nutritional and hormonal requirements of the tissues. But this quickly gave place to a period during which cultured tissues were used as a research tool, in studying more general problems of plant cell physiology and biochemistry and the complex processes of differentiation and organogenesis. The achievements were considerable; but above all, the finding that whole plants could be regenerated from undifferentiated tissues – even single cells – in culture gave the method enormous power. In an extraordinary way this has meant that at one point in time the entity – a plant – can be handled like a microorganism and subjected to the rigorous procedures of molecular biology, and at another time called almost magically back into existence as a free-living, macroscopic organism. The implications and applications of this finding are currently being explored in many contexts, not least in the field of practical application. If genetic engineering, involving the direct manipulation of the stuff of heredity, is ever to contribute to that part of man's welfare that depends on his exploitation of plants, the procedures adopted will inevitably depend ultimately upon the recovery of "real" plants from cultured components. No wonder, then, that the technology has escaped from

the confines of the university laboratory to become part of the armory of industry and agriculture!

Yet, notwithstanding the wide interest in the methods of plant tissue culture, the range of modern techniques has never hitherto been treated comprehensively in one text. Both novitiate and initiate have had to explore the large and scattered literature to unearth procedures appropriate to their interest. This volume makes good the deficiency, for the experiments described cover almost every aspect of the tissue culture art. But this is far more than any cookery book. The methods of achieving growth, cell division, and morphogenesis in vitro are set in their appropriate contexts. The chapters not only describe how to carry out procedures, but offer lucid accounts of the historical background and interpretations of the results likely to be obtained, backed up by extensive bibliographies. The authors are pecularily well fitted to have writen such a text, with their extensive experience of the application, development, and teaching of tissue culture methods. Directed in the first instance toward students, their treatment of the topics will prove of immense value to a much wider range of readers, whatever their previous knowledge or field of potential application.

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PREFACE TO THE SECOND EDITION

The need for a new edition of a scientific book is directly related to the influx of new ideas and developments into the field. Solid gains have been made in the past few years in the applications of plant tissue culture techniques to agriculture and industry, and we would like to share some of this information with our readers. Another obvious reason for publishing a new edition is to rectify any errors and misinformation that may have silently crept into the original text.

Although a few organizational changes have been made, the general format of the book remains the same. We have complied with requests to enlarge the section on terminology and definitions. Additional information has been provided on equipment and facilities in Chapter 2. An introduction to the techniques used for the preservation of germplasm will be found in Chapter 15. Since it is hoped that this book will give the reader a broad introduction to the field, we have included a brief nonexperimental chapter on two special topics: virus eradication and plant tumors and genetic engineering. A table at the end of the book gives the formulations of some tissue culture media: Murashige and Skoog's, Gamborg's B5, White's, and Schenk and Hildebrandt's.

The manuscript of the book was completed by LWR during an appointment as a Senior Visiting Researcher to Queen Elizabeth College, University of London. He gratefully acknowledges the cooperation of Professor P. B. Gahan, Head of the Biology Department.

We are gratefui to all reviewers, colleagues, and students who have assisted us by their constructive criticism and suggestions.

PREFACE TO THE FIRST EDITION

The purpose of this book is to introduce a basic experimental method for each of the major areas of investigation involving the isolation and culture of plant cells, tissues, and organs. Each chapter is devoted to a separate aspect of plant tissue culture, and the chapters are arranged, in general, in order of increasing technical complexity. Although the book was written mainly for use by college undergraduates, research workers from various botanical fields and biology students in high school will also find the text within their grasp. In view of the diverse laboratory facilities that may be available, the experiments selected require a minimum of special equipment. A list of suppliers is given at the end of the book. The book is designed as a laboratory textbook for a course on plant tissue culture techniques, although it may also be used as a supplementary text for developmental botany and biology courses.

The opening chapters present a brief historical survey of the field of plant tissue culture, and a background in sterilization and aseptic techniques. The third chapter examines the various components of the nutrient medium, including inorganic salts, vitamins and other organic supplements, carbohydrates, plant growth regulators, media matrices, and instructions for the preparation of a typical nutrient medium.

The remainder of the text involves laboratory experiments using special culture procedures, and each chapter follows the same format: purpose of the experiment and background information, list of materials, procedure, results, questions for discussion, and selected references. Most of the chapters have an appendix giving additional experiments and techniques. The opening experimental chapters describe the initiation and maintenance of a callus culture, and the preparation of a suspension or liquid culture. Students will repeat White's classic experiment involving the unlimited growth of isolated tomato roots. Another chapter outlines methods for the induction of tracheary element differentiation in cultured tissues. Several chapters introduce diverse approaches to

plant propagation by in vitro techniques. The student will attempt to regenerate *Coleus* plants from isolated leaf discs following the cultural principles of organogenesis. The development of embryoids is observed in a suspension culture of carrot cells, and this procedure demonstrates another approach to plant propagation from somatic tissue. A method for the clonal reproduction of plants by the culture of the shoot apex is provided. Isolated protoplasts are prepared and directions given for the induction of protoplast fusion with the creation of somatic hybrids. An important tool in plant breeding involves anther culture, with the resulting production of monoploid plantlets bearing a single set of chromosomes. A brief chapter introduces the concept of using tissue cultures for the commercial production of chemical and medicinal compounds. And finally, some quantitative methods of expressing the results of in vitro experiments are given.

The authors wish to acknowledge the valuable comments received during the preparation of the manuscript, and we particularly thank M. Davey, N. P. Everett, T. Ford, W. P. Hackett, G. G. Henshaw, J. Heslop-Harrison, M. G. K. Jones, A. Komamine, J. O'Hara, V. Raghavan, T. A. Thorpe, J. G. Torrey, G. Wilson, L. A. Withers, and M. M. Yeoman for critical review of the contents. We also wish to thank Miss H. Bigwood and Mr. A. Pugh for their artistic and photographic assistance. The generosity of colleagues who have provided us with original plates and negatives for illustrations is acknowledged with the figures.

Most of LWR's contributions to this book were written during an appointment as a Visiting Fellow and Fulbright Senior Scholar to the Australian National University, Canberra. He gratefully acknowledges the excellent cooperation of the Botany Department, the Australian National University, the Research School of Biological Sciences, and the Australian-American Educational Foundation. The authors are also indebted to Florence Roberts for her editorial suggestions.

TERMS, ABBREVIATIONS, AND SYNONYMS

ABA	abscisic acid
adenine	aminopurine; exhibits cytokinin activity in bud initiation
adventitious	initiation of a structure out of its usual place, i.e., arising sporadically. Adventitious roots can originate from leaf or stem tissue
aseptic	sterile; free from contamination by microorganisms
autotropy	self-sufficiency, e.g., an auxin-autotropic organism synthesizes its own supply of auxin
auxin	plant growth regulator stimulating shoot cell elongation and resembling IAA in physiological activity
axenic	aseptic
B5	Gamborg et al. (1968) medium
BAP	benzylaminopurine; BA; synthetic cytokinin
batch culture	cell suspension grown in a fixed volume of liquid medium; example of a closed culture
benazolin	4-chloro-2-oxybenzothiazolin-3-yl acetic acid; an auxin
boring platform	sterile bottom half of a Petri dish used for preparing explants with a cork borer
C	symbol for explant cutting guide
C_4 plants	plants having the C_4 dicarboxylic acid pathway
C_3 plants	plants fixing CO_2 directly through the reductive pentose phosphate pathway
callus	disorganized meristematic or tumorlike mass of plant cells formed under in vitro conditions
caulogenesis	initiation of one or more shoot primordia
cell cycle	sequence of events occuring during cell division, and measured by the time interval between one of these events and a similar event in the next cell generation. The demonstrable phases include mitosis (M) and DNA synthesis (S). The time period between S and M is termed G_2, whereas G_1 represents the interval between M and S.
cellulase Onozuka (R-10, RS)	cellulose-degrading enzyme derived from *Trichoderma viride*; also contains other enzymatic activities (E.C. 3.2.1.4)

Cellulysin	cellulase preparation isolated from *Trichoderma viride*; hydrolyzes β-1,4-glucan linkages in cellulose (E.C. 3.2.1.4)
chemostat	instrument for maintaining an open continuous culture; growth rate and cell density are maintained constant by regulating the input of a growth-limiting nutrient
chromic acid	aqueous solution of chromium trioxide
clone	genetically identical organisms propagated from a single individual plant
closed continuous culture	suspension culture in which the influx of fresh liquid medium equals the efflux of spent medium; all cells are retained within the system
continuous culture	suspension culture continuously supplied with an influx of fresh medium and maintained at a constant volume
cybrid	cytoplasmic hybrid; heteroplast
cytokinin	plant growth regulator stimulating cell division and resembling kinetin in physiological activity; mainly N^6-substituted aminopurine compounds
d	density of culture
DDH_2O	double-distilled water
DEGS	diethylene glycol succinate
DF	dilution factor
dicamba	3,6-dichloro-O-anisic acid; an auxin
2,4-D	2,4-dichlorophenoxyacetic acid; an auxin
6-[γ,γ-dimethylallylamino] purine	N^6-[Δ^2-isopentyl]-adenine; IPA; a cytokinin
DNA	deoxyribonucleic acid
Driselase	enzyme preparation from Basidiomycetes containing laminarinase, xylanase, and cellulase activities
embryoid	embryolike structure formed under in vitro conditions; structure has potential for further development into a plantlet
explant	excised fragment of plant tissue or organ used to start a tissue culture; primary explant
F	symbol for Whatman No. 1 filter paper used as a blotter during the culture procedure
fermenter	instrument for culturing a cell suspension under batch or continuous culture conditions
friable	crumbles or fragments readily
genetic engineering	use of a vector (e.g., plasmid) for the transfer of genetic information
gibberellin	plant growth regulator with physiological activity similar to GA_3 (gibberellic acid)
GLC	gas–liquid chromatography
glycerol	glycerin

glycine	aminoacetic acid
habituation	changes in exogenous nutritional requirements occurring during culture; anergy
haploid	having a single set of chromosomes; monoploid
hardening	application of mild environmental stress to prepare the plant for more rigorous growing conditions, e.g., plantlets are given greater illumination, lower nutrients, and less moisture to enhance their survival outside of the culture tube
hemicellulase (Sigma)	enzyme preparation from *Aspergillus niger* liberating D-galactose from hemicellulose; also contains cellulase activity
heterokaryon	fusion of unlike cells with dissimilar nuclei present; heterokaryocyte
heteroplast	cell containing foreign organelles; cytoplasmic hybrid
homokaryon	fusion of similar cells
IAA	indole-3-acetic acid; a naturally occurring auxin
inositol	*myo*-inositol; *meso*-inositol; *i*-inositol
in vitro	culture of living material literally "in glass," i.e., on an artificial medium and under aseptic conditions
in vivo	processes occurring within the intact living organism
IPA	6-[γ,γ-dimethylallylamino] purine; N^6-[Δ^2-isopentyl]-adenine; a cytokinin
K	kinetin; N^6-furfuryladenine; a synthetic cytokinin
Macerase	preparation from *Rhizopus* showing polygalacturonase (E.C. 3.2.1.15) activity
Macerozyme R-10	preparation from *Rhizopus* containing polygalacturonase (E.C. 3.2.1.15), pectin transeliminase (E.C. 4.2.2.2), hemicellulase, and other unknown maceration factors
Meicelase (CESB, CMB)	preparation from *Trichoderma viride* containing cellulase (E.C. 3.2.1.4)
meristem culture	apical meristem culture; explant consisting only of apical dome tissue distal to the youngest leaf primordium
meristemoid	cluster of meristematic cells within a callus with the potential to form a primordium
MI	mitotic index
MS	Murashige and Skoog's (1962) medium; same as Linsmaier and Skoog's (1965) medium in mineral composition, although differing in vitamin supplement
mutagen	chemical or physical treatment capable of inducing gene mutation
NAA	α-naphthaleneacetic acid; a synthetic auxin
NaFeEDTA	ferric ethylenediamine tetraacetate, sodium salt of; employed for maintaining iron in solution
nicotinic acid	niacin
O.D.	outside diameter

open continuous culture	suspension culture in which the influx of fresh liquid medium is equal to the efflux of culture, i.e., the cells are flushed out with the spent medium
organoid	anomalous organlike structure arising from a tissue culture
osmoticum	isotonic plasmolyticum; external medium of low osmotic potential that approximates the concentration of solutes dissolved within the cell vacuole; prevents the bursting of naked protoplasts due to excessive water uptake
passage time	interval between successive subcultures
PCV	packed cell volume
pectinase	polygalacturonase; enzyme liberating galacturonic acid from polygalacturonic acid (E.C. 3.2.1.15)
Pectolyase	Pectolyase Y-23; enzyme preparation from *Aspergillus japonicus* reported to contain endopolygalacturonase (E.C. 3.2.1.15), endopectin lyase (E.C. 4.2.2.3), and an unknown maceration factor
PEG	polyethylene glycol
plantlet	miniature plant with root and shoot system regenerated by tissue culture techniques
primordium (pl primordia)	earliest detectable stage of differentiation of a cell or organ, e.g., leaf or root primordium
protoplast	living isolated plant cell following removal of cell wall either by enzymatic or mechanical method
pyridoxine	vitamin B_6
reversal transfer	transfer of a culture from a callus-supporting medium to a shoot-inducing medium
rhizogenesis	initiation of one or more adventitious root primordia
Rhozyme HP-150 concentrate	enzyme preparation hydrolyzing the class of polysaccharides known as gums or mucilages that contain hexose and/or pentose polymers
RNA	ribonucleic acid
sector inoculum	fragment of main root and lateral roots used to start a root subculture
shoot-ap . culture	explant consisting of apical dome plus a few subjacent leaf primordia
somatic hybrid	hybrid cell or organism produced asexually, e.g., by the fusion of two protoplasts
S-phase	period of cell cycle involving DNA synthesis
subculture	asceptic transfer of part of a culture (inoculum) to a fresh medium; passage
synchronous culture	cycles of individual cells that have been brought into phase or synchrony, i.e., they pass through the sequential events of the cell cycle at the same time
synkaryocyte	hybrid cell produced by fusion of nuclei in a heterokaryon

TE	tracheary element(s)
thiamine	vitamin B_1
tissue culture	cellular mass grown in vitro on solid medium or supported and nurtured with liquid medium; the cells are in protoplasmic continuity
totipotency	ability to regenerate an entire organism from a single cell or plant part
2,4,5-T	2,4,5-trichlorophenoxyacetic acid; synthetic auxin
transfer	see subculture
turbidostat	instrument for growing an open continuous culture into which fresh medium flows due to changes in culture turbidity, i.e., cell density
UV	ultraviolet light
vit	vitamin(s)
v/v	percent "volume in volume"; number of cubic centimeters of a constituent in 100 cm^3 of solution
w/v	percent "weight in volume"; number of grams of constituent in 100 cm^3 of solution

1

Culture of plant cells, tissues, and organs

Early attempts, 1902–1939

The concept that the individual cells of an organism are totipotent is implicit in the statement of the cell theory. Schwann (1839) expressed the view that each living cell of a multicellular organism should be capable of independent development if provided with the proper external conditions (White, 1954). A totipotent cell is one that is capable of developing by regeneration into a whole organism, and this term was probably coined by Morgan in 1901 (Krikorian and Berquam, 1969). The basic problem of cell culture was clearly stated by White (1954). If all of the cells of a given organism are essentially identical and totipotent, then the cellular differences observed within an organism must arise from responses of those cells to their microenvironment and to other cells within the organism. It should be possible to restore suppressed functions by isolating the cells from those organismal influences responsible for their suppression. If there has been a loss of certain functions, so that the cells in the intact organism are no longer totipotent, then isolation would have no effect on restoring the lost activities. The use of culture techniques enables the scientist to segregate cells, tissues, and organs from the parent organism for subsequent study as isolated biological units. The attempts to reduce an organism to its constituent cells, and subsequently to study these cultured cells as elementary organisms, is therefore of fundamental importance (White, 1954).

Several plant scientists performed experiments on fragments of tissue isolated from higher plants during the latter part of the nineteenth century. Wound callus formed on isolated stem fragments and root slices was described (Trécul, 1853; Vöchting, 1878; Rechinger, 1893). Callus refers to a disorganized proliferated mass of actively dividing cells. Rechinger (1893) examined the "minimum limits" of divisibility of isolated fragments of buds, roots, and other plant material. Although no nutrients

were used in these experiments, he concluded that pieces thicker than 1.5 mm were capable of further growth on sand moistened with water. Since isolated fragments thinner than 1.5 mm were apparently incapable of further development, he concluded that this was the size limit beneath which the tissue lost the capability of proliferation. Rechinger reported that the presence of vessel elements appeared to stimulate growth of the fragments. Unfortunately, he did not pursue this clue, since his observations suggested the proliferative ability of cambial tissue was associated with vascular tissues (Gautheret, 1945).

Haberlandt (1902) originated the concept of cell culture and was the first to attempt to cultivate isolated plant cells in vitro on an artificial medium. A tribute to Haberlandt's genius with a translation of his paper "Experiments on the culture of isolated plant cells" has been published (Krikorian and Berquam, 1969). Unlike Rechinger, Haberlandt believed that unlimited fragmentation would not influence cellular proliferation. The culture medium consisted mainly of Knop's solution, asparagine, peptone, and sucrose. Although the cultured cells survived for several months, they were incapable of proliferation. Haberlandt's failure to obtain cell division in his cultures was, in part, due to the relatively simple nutrients and to his use of highly differentiated cells. Since Haberlandt did not use sterile techniques, it is difficult to evaluate his results, because of the possible effects of bacterial contamination (Krikorian and Berquam, 1969). As examples of his genius, Haberlandt suggested the utilization of embryo sac fluids and the possibility of culturing artificial embryos from vegetative cells. In addition, he anticipated the paper-raft technique (Muir, 1953). Following his lack of success with cell cultures, Haberlandt became interested in wound healing. Experiments in this area led to the formulation of his theory of division hormones. Cell division was postulated as being regulated by two hormones. One was "leptohormone," which was associated with vascular tissue, particularly the phloem. The other was a wound hormone released by the injured cells. Subsequent research investigators (Camus, 1949; Jablonski and Skoog, 1954; Wetmore and Sorokin, 1955) verified the association of hormones with vascular tissues.

Early in the twentieth century interest shifted to the culture of meristematic tissues in the form of isolated root tips. These represented the first aseptic organ cultures. Robbins was the first to develop a technique for the culture of isolated roots (1922a,b) and Kotte, a student of Haberlandt's, published independently similar studies (1922a,b). These cultures were of limited success. Robbins and Maneval (1923), with the aid

of subcultures, maintained maize roots for 20 weeks. White (1934), experimenting with tomato roots, succeeded for the first time in demonstrating the potentially indefinite culture of isolated roots. According to White (1951), two difficulties hampered the development of a successful method for culturing excised plant material between 1902 and 1934: (a) the problem of choosing the right plant material, and (b) the formulation of a satisfactory nutrient medium. With the introduction of root tips as a satisfactory experimental material, the crucial problem became largely one of organic nutrition. White's early success with tomato roots can be attributed to his discovery of the importance of the B vitamins, plus the fact that indefinite growth was achieved without the addition of any cell-division factor to the liquid medium.

It is important at this point to make a distinction between an organ culture and a tissue culture. In the case of excised roots as an example of an organ culture, the cultured plant material maintains its morphological identity as a root with the same basic anatomy and physiology as in the in vivo roots of the parent plant. There are some exceptions, and slight changes in anatomy and physiology may occur during the culture period. According to Street (1977a), the term "tissue culture" can be applied to any multicellular culture growing on a solid medium (or attached to a substratum and nurtured with a liquid medium) that consists of many cells in protoplasmic continuity. Typically, the culture of an explant, consisting of one or more tissues, results in a callus that has no structural or functional counterpart with any tissue of the normal plant body.

The first plant tissue cultures, in the sense of long-term cultures of callus, involved explants of cambial tissues isolated from carrot (Gautheret, 1939; Nobécourt, 1939) and tobacco tumor tissue from the hybrid *Nicotiana glauca* × *N. Langsdorffii* (White, 1939). The latter tumor tissue requires no exogenous cell-division factor. Results from these three laboratories, published independently, appeared almost simultaneously. Fortunately, plant physiologists working in other areas had discovered some of the hormonal characteristics of indole-3-acetic acid, IAA (Snow, 1935; Went and Thimann, 1937), and the addition of this auxin to the culture medium was essential to the success of the carrot cultures maintained by Nobécourt and Gautheret. According to Gautheret (1939), the carrot cultures required Knop's solution supplemented with Bertholot's salt mixture, glucose, gelatine, thiamine, cysteine-HCl, and IAA (see White, 1941). The goal at that time was to demonstrate the potentially unlimited growth of a given culture, by repeated subcultures, with

the formation of undifferentiated callus. The workers were fascinated by the apparent immortality of their cultures and devoted much effort to determining the nutritional requirements for sustained growth.

Basic studies on nutrition and morphogenesis, 1940–1978

Because of the lull in botanical research during the war years (1939–45), relatively little was accomplished until a resurgence of interest in the early 1950s. Some fundamental studies, however, were undertaken. White and Braun (1942) initiated experiments on crown gall and tumor formation in plants. Probably the most significant event leading to advancement in the next decade was the discovery of the nutritional quality of liquid endosperm extracted from coconut (coconut milk or water). Following the success of Van Overbeek and his colleagues (1941) with the culture of isolated *Datura* embryos on a medium enriched with coconut milk, other workers rapidly adopted this natural plant extract. The combination of coconut milk and 2,4-D had a remarkable effect on the proliferation of cultured carrot and potato tissues (Caplin and Steward, 1948; Steward and Caplin, 1951; 1952). Although it was first thought that a single substance, termed the coconut milk factor, was involved as a growth stimulant, several constituents were later found responsible for its activity. Steward's group at Cornell University made numerous contributions in technique, nutrition, quantitative analyses of culture growth, and morphogenesis. The regeneration of carrot plantlets from cultured secondary-phloem cells of the taproot clearly demonstrated the totipotency of plant cells (Krikorian, 1975). The phenomenon of somatic embryogenesis in carrot cultures was discovered at approximately the same time by Steward (1958) and Reinert (1959). The consequences of this discovery will be discussed in Chapter 11.

The discovery of cytokinins stems from Skoog's tissue culture investigations at the University of Wisconsin. During attempts to induce unlimited callus production from mature tobacco pith cells, numerous compounds were tested for possible activity in stimulating cell division. Although coconut milk or yeast extract plus IAA promoted cell division, efforts were made to locate a specific cell-division factor. Since adenine, in the presence of auxin, was found to be active in stimulating callus growth and bud formation in tobacco cultures (Skoog and Tsui, 1948; Sterling, 1950), nucleic acids were then examined. Skoog's group eventually located a potent cell-division factor in degraded DNA preparations. It was isolated, identified as 6-furfurylaminopurine, and named

kinetin (Miller et al., 1955). The related analogue, 6-benzylaminopurine, was then synthesized, and it too stimulated cell division in cultured tissues. The generic term cytokinin was given to this group of 6-substituted aminopurine compounds that stimulate cell division in cultured plant tissues and behave in a physiological manner similar to kinetin. Later it was discovered that zeatin, isopentyl adenine, and other cytokinins are naturally occurring plant hormones. Often these compounds are attached to ribose sugar (ribosides) or to ribose and phosphate (ribotides). The stimulatory properties of coconut milk are partly due to the presence of zeatin riboside. Skoog and Miller (1957) advanced the hypothesis that shoot and root initiation in cultured callus can be regulated by varying the ratio of auxin and cytokinin in the medium (see chap. 6). In addition to the cytokinins, other endogenous cell-division factors may exist in plant tissues (Wood et al., 1969).

It was found that callus fragments, transferred to a liquid medium and aerated on a shaker, gave a suspension of single cells and cell aggregates that could be propagated by subculture (Muir, 1953; Muir, Hildebrandt, and Riker, 1954). Steward's group made extensive use of carrot suspension cultures, and it became evident that this technique offered much potential for studying many facets of cell biology and biochemistry (Nickell, 1956). Street and co-workers have pioneered the development of various procedures for the culture of cell suspensions (e.g., chemostats and turbidostats; see chap. 9). Torrey and his colleagues also conducted studies on cell suspensions (Torrey and Shigomura, 1957; Torrey and Reinert, 1961; Torrey, Reinert, and Merkel, 1962).

Muir (1953) succeeded in developing a technique for the culture of single isolated cells. Single cells were placed on squares of filter paper, and the lower surface of the paper was placed in contact with an actively growing "nurse" culture. This paper-raft nurse technique provided the isolated cells not only with nutrients from the medium via the older culture, but also with growth factors synthesized by the nurse tissue. Although Muir's experiments involved bacteria-free crown gall (*Agrobacterium tumefaciens*) tumor cells, single-cell clones were produced later from normal cells. In another approach, a single cell was suspended as a hanging drop in a microchamber (Torrey, 1957; Jones et al., 1960). The agar-plating method of Bergmann (1960) involved separating a single-cell fraction by filtration, mixing the cells with warm agar, and then plating the cells as a thin layer in a Petri dish. These early investigations with single-cell cultures have been reviewed by Street (1977b) and Hildebrandt (1977). Although Muir and his colleagues reported in 1954

that single isolated cells exhibited cell division, this claim was contes by De Ropp (1955). Subsequent investigations provided the necess evidence that single cells are capable of proliferation (Torrey, 1957; M et al., 1958). The question of totipotency was completely resolved Vasil and Hildebrandt (1965) by the demonstration that a single isolat cell can divide and ultimately give rise to a whole plant.

Plant tissue cultures have been used extensively for the study of c todifferentiation, particularly the formation of tracheary elements in c tured tissues. A variety of different techniques have been employe Wedges of agar containing auxin and sucrose "grafted" to a block of call induced tracheary element formation (Wetmore and Sorokin, 1955; We more and Rier, 1963; Jeffs and Northcote, 1967). Primary explants fro many different plant tissues are capable of producing tracheary elemen during culture on agar or in a liquid medium (see chap. 7). A rece symposium was held in Edinburgh on differentiation in vitro (Yeoma and Truman, 1982).

Many of the early investigators employed either herbaceous or wood dicot tissues as sources of primary explants, although other groups c plants were also used as experimental material. Morel (1950) successfull cultured monocot tissues with the aid of coconut milk. Ball (1955) cu tured tissues of the gymnosperm *Sequoia sempervirens*, and Tulecke pre pared haploid cultures from the pollen of *Taxus* (Tulecke, 1959) an *Ginkgo biloba* (Tulecke, 1953; 1957). Harvey and Grasham (1969) pub lished media requirements for establishing cultures of 12 conifer species Tissue culture procedures have been used in developmental anatom studies involving excised shoot apices of lower plants (e.g., ferns, *Sela ginella*, and *Equisetum*; see Wetmore and Wardlaw, 1951).

During the 1960s it was shown that cultured pollen and the micro- sporogenous tissue of anthers have the potential to produce vast numbers of haploid embryos (Guha and Maheshwari, 1966, 1967; Bourgin and Nitsch, 1967). Later, with a technique developed by C. Nitsch, it became possible to culture microspores of *Nicotiana* and *Datura*, to double the chromosome number of the microspores, and to collect seeds from the homozygous diploid plants within a 5-month period (Nitsch, 1974, 1977). Although there are technical problems associated with this tech- nique, haploid cultures have been used successfully in China for the selection of improved varieties of crop plants (see chap. 14).

Another important development during the 1960s was the enzymatic isolation and culture of protoplasts (Cocking, 1960). This method in- volves removing the cell wall with purified preparations of cellulase and

pectinase, while regulating protoplast expansion with an external osmoticum. The cultured protoplasts regenerate new cell walls, form cell colonies, and ultimately form plantlets (Takebe, Labib, and Melchers, 1971). Some of the experimental approaches currently employed include (a) protoplast fusion within species, between species, between monocots and dicots, and even between plants and animals; (b) introduction of mitochondria and plastids into protoplasts; (c) uptake of blue-green algae, bacteria, and viruses by protoplasts; and (d) the transfer of genetic information into isolated protoplasts. Cells containing foreign organelles are termed cytoplasmic hybrids or heteroplasts, whereas cells containing transferred nuclei are referred to as heterokaryons or heterokaryocytes. The fusion of the two nuclei in heterokaryons produces hybrid cells (see chap. 13).

Plant tissue culture techniques have been widely used for the commercial propagation of plants. Most of the applications are based on the characteristic of cytokinins to stimulate bud proliferation in the cultured shoot apex. Ball (1946) demonstrated the possibility of regenerating plants from isolated explants of angiosperm shoot apices. Later, Wetmore and Morel regenerated whole plants from shoot apices measuring 100–250 μm in length and bearing one or two leaf primordia (see Wetmore and Wardlaw, 1951). Modifications of Morel's (1960) shoot-apex technique have been used for orchid propagation (Morel, 1964). Premixed culture media, specifically formulated for the propagation of certain plants, are commercially available (see Commercial sources of supplies). Several recent publications are devoted to technical problems associated with the mass propagation of higher plants (Vasil, 1980; Conger, 1981; Constantin et al., 1981; Thorpe, 1981; Bonga and Durzan, 1982; Tomes et al., 1982). This subject will be discussed further in Chapter 10.

One of the earliest applications of plant tissue culture involved the study of plant tumor physiology. White and Braun (1942) reported the growth of bacteria-free crown-gall tissue. Braun has devoted his career at Rockefeller University to the study of plant cancer (Braun, 1974, 1975), and a summary of this research has appeared (Butcher, 1977). Related to these studies is the phenomenon of "habituation." Gautheret (1946) observed that a callus culture of *Scorzonera hispanica*, which originally required auxin in the medium for growth, often developed outgrowths of callus that would grow indefinitely on an auxin-deficient medium. The term habituation refers to inherited changes in nutritional requirements arising in cultured cells, especially changes involving plant hormones. For example, an auxin-habituated culture has lost its original

requirement for exogenous auxin (Butcher, 1977). These cultures are important in investigations of plant cancer. The grafting of auxin- and cytokinin-habituated tissues into healthy plants produces tumors (see Butcher, 1977). Tissue culture techniques have also been used to produce pathogen-free plants via apical meristem cultures. Ingram and Helgeson (1980) reviewed the applications of in vitro procedures to plant pathology.

An interesting account of the early development in both plant and animal cell culture is given in White's book (1954), and Gautheret's monumental work (1959) was an invaluable guide to the early investigators. A summary of the pioneer studies on plant morphogenesis involving cultured tissues was compiled by Butenko (1964). Recently Gautheret (1983) recalled some of the highlights of the field.

Emergence of a new technology, circa 1978 to the present

By the late 1970s it became evident that plant tissue culture technology was beginning to make significant contributions to agriculture and industry (Murashige, 1978; Zenk, 1978). In agriculture, the major areas are haploid breeding, clonal propagation, mutant cultures, pathogen-free plants, production of secondary products, and genetic engineering. In addition, the cryopreservation of plant tissue cultures and the establishment of in vitro gene banks have attracted considerable interest (see chap. 15).

The greatest success has been achieved with in vitro clonal propagation. In vitro techniques have revitalized the orchid industry. Murashige (1977) estimated that more than 600 species of ornamental plants have been cloned. Cloning has been extended to forest trees, fruit trees, oil-bearing plants, vegetables, and numerous agronomic crop plants. Clonal propagation of potato plants has been achieved on a large scale by the regeneration from isolated leaf-cell protoplasts (Shepard, 1982). By 1982 more than 100 tissue culture facilities were engaged in the commercial propagation of plants (Loo, 1982). In other areas, plant tissue culture has had only limited economic success. Haploid breeding has produced relatively few established cultivars, mainly because of the low frequency of the appearance of new agriculturally important genotypes by this method. Progress is being made, however, particularly in China (Loo, 1982). The application of mutagenic agents to cultures, followed by suitable screening techniques, has led to the regeneration of mutant plants showing disease or stress resistance. Chaleff (1983) reviewed the tech-

nical problems associated with this approach. Several pathogen-free plants have been developed (Murashige, 1978), and tissue culture technology now plays an important role in plant pathology. For example, protoplasts are currently employed for the study of virus infection and biochemistry (Rottier, 1978). The prospects of success with the genetic manipulation of plants have created considerable public interest. Yet advances to date have been largely theoretical. Two approaches may be considered. The first technique involves the transfer of genetic information by the fusion of protoplasts isolated from two different organisms. This method provides the opportunity of producing hybrids between related but sexually incompatible species. Melchers and his colleagues (1978) produced a somatic hybrid plant from the fusion of potato and tomato protoplasts, both members of the Solanaceae family. It appears unlikely, however, that this technique will yield any plants of economic importance in the near future. The second technique concerns the insertion of foreign genes attached to a plasmid vector into the naked protoplast (Barton and Brill, 1983; Dodds and Bengochea, 1983). The transfer of the plasmid DNA into the protoplast is usually achieved by means of liposomes. A major problem, however, is whether the inserted gene will be integrated into the host genome, transcribed, and expressed in the mature plant. The final problem is the regeneration of whole plants from the transformed single cells. Several research groups have produced transformed tobacco plants following single-cell transformation (i.e., gene insertion) (Wullems et al., 1982; Chilton, 1983).

Industrial applications involve large-scale suspension cultures capable of synthesizing significant amounts of useful compounds. These secondary products of industrial interest include antimicrobial compounds, antitumor alkaloids, food flavors, sweeteners, vitamins, insecticides, and enzymes. A major problem has been the genetic instability of the cultures, as well as engineering problems associated with this technique. Nevertheless, progress has been made at the industrial level, especially by the Japanese (Misawa, 1977; 1980). Further discussion of this topic will be found in Chapter 16.

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2

A plant tissue culture laboratory

A laboratory devoted to in vitro procedures with plant tissues must have adequate space for the performance of several functions. It must provide facilities for (1) media preparation, sterilization, cleaning, and storage of supplies; (2) aseptic manipulation of plant material; (3) growth of the cultures under controlled environmental conditions; (4) examination and evaluation of the cultures; and (5) assembling and filing of records (White, 1963). The grouping of functions will vary considerably from one laboratory to another. According to White (1963), the ideal organization will allow a separate room for each of the following functions: media preparation, aseptic procedures, incubation of cultures, and general laboratory operations. If one has the opportunity to plan an in vitro laboratory in advance, the component facilities should be arranged as a production line (Street, 1973). The area involved with washing and storage of glassware should lead to the facilities for oven sterilization and media preparation. Materials should then move from autoclave sterilization to the aseptic transfer facility. After the aseptic operations, the cultures are transferred to incubators or controlled-environment chambers. The cultures should be in close proximity to the laboratory containing microscopes and facilities for evaluation of the results. Discarded and contaminated cultures are transferred back to the washing area. It is of the utmost importance to give careful consideration to the arrangement of the aseptic procedures. Because some laboratories do not have a separate sterile room, some type of laminar flow cabinet or bacteriological glove box is required. This facilitiy must be located in an area free from drafts and with a minimum of traffic. In addition, it should not be located in the vicinity of scientists from other research groups working with airborne microorganisms (see chap. 3).

Dishwashing. Discarded cultures, as well as contaminated ones, are autoclaved briefly in order to liquefy the agar and to kill any contaminants

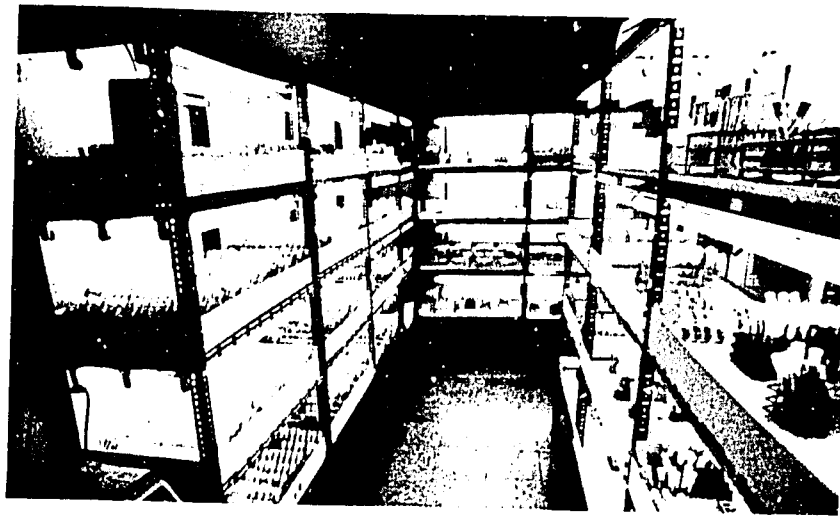
that may be present. The culture glassware is easier to wash after the spent medium has been liquefied and removed. After scrubbing with a brush in a hot detergent bath, the glassware is rinsed repeatedly with tap water, and then given two or three rinses in distilled water (de Fossard, 1976). If an automatic dishwashing machine is used, a final rinse with distilled or demineralized water should be used to remove any possible traces of detergent. After washing, the glassware is oven dried prior to storage. Certain cell cultures require scrupulously clean culture vessels; therefore, a routine dishwashing program is inadequate. New glassware may release chemicals that are toxic to the cultured tissues. Additional information can be obtained from Street (1973) and Biondi and Thorpe (1981).

Media preparation. Although media preparation requires a balance sensitive to milligram quantities for weighing hormones and vitamins, a less sensitive scale may be used for weighing agar and carbohydrates. The media reagents should be shelved near the balance for convenience. A refrigerator in the media room is necessary for storing stock solutions and chemicals that degrade at room temperature. A combination hot plate and magnetic stirrer is a time saver for dissolving inorganic reagents. Either a pH meter or pH indicator paper is required for adjusting the final pH of the medium. Relatively large quantities of single- and double-distilled water must be available in the media room. Sterilization equipment is an integral part of media preparation. A commercial electric stove is the most economical type of oven sterilization. Wet-heat sterilization involves either an autoclave or a pressure cooker. Some hormones and vitamins are sterilized by ultrafiltration at room temperature. After sterilization of the culture vessels by dry heat and autoclaving the medium, the culture tubes are poured in the transfer chamber. The subsequent aseptic techniques will be discussed in Chapters 3 and 4.

Incubation of the cultures. The freshly prepared cultures are grown under carefully regulated environmental conditions, i.e., temperature, light, and humidity. This is accomplished with an incubator, plant growth chamber, or controlled environment room (Fig. 2.1). If cell suspensions are cultured, some type of shaker or aeration equipment will be necessary (Fig. 2.2). Several engineering aspects should be considered in designing a culture room: safety and convenience of the electrical system; air flow for uniform temperature regulation; arrangement of the shelving; and elimination of airborne contaminants (Wetherell, 1982). Optimal envi-

ronmental conditions will vary depending on the species and the purpose of the experiment, and consideration should be given to diurnal temperature variations, light intensity, light quality, and photoperiod (relative length of light–dark cycles). Fluorescent lamps have certain advantages over incandescent sources. The former have a better spectral quality, a more convenient shape, and a lower heat output than incandescent bulbs. Some cultures, however, appear to show the best growth in the presence of a mixture of both types of illumination. Experiments conducted by Murashige (1974) with *Asparagus*, *Gerbera*, *Saxifraga*, and bromeliads indicated an optimum light intensity of 1,000 lux during culture initiation and shoot proliferation. A higher optimum of 3,000–10,000 was required for the establishment of plantlets. These experiments utilized Gro-Lux or white fluorescent lamps with a daily exposure period of 16 hr (Murashige, 1974). It is advisable to equip the culture room with a clock-operated timing switch for the regulation of photoperiods. Although some investigators may want to expose the cultures to thermoperiodic cycles, most experiments are conducted with constant temperatures set at approximately 25–27°C. Some morphogenetic responses are evidently sensitive to temperature fluctuations (see Murashige, 1974). Additional information on the effects of various light sources on plant growth can be found in the review by Cathey and Campbell (1982).

Fig. 2.1. A walk-in controlled environment room for incubation of in vitro cultures. (International Potato Center.)

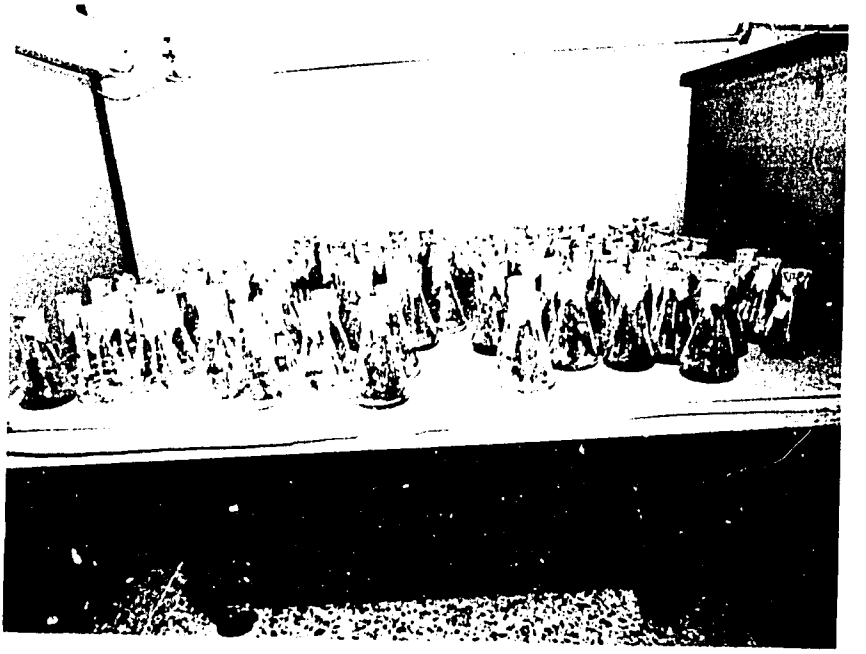


Additional needs. General laboratory requirements vary considerably, depending on the type of data required. A suitable hand lens and dissection microscope are important for the macroexamination of the cultures, and a compound microscope equipped with photomicrograph accessories is available in most laboratories. A chemical hood should be used with maceration procedures involving chromic acid, as well as for the storage of volatile and potentially dangerous chemicals. Protoplast purification requires a bench-top centrifuge. All plant tissue culture laboratories should be equipped with a fire extinguisher and a first aid kit.

Descriptions of the facilities used by the early investigators can be found in the publications of Gautheret (1959), White (1963), and Butenko (1964). Street (1973), Wetherell (1982), Bonga (1982), de Fossard (1976), and Biondi and Thorpe (1981) describe modern laboratory facilities.

Possibly the best way of deciding on the type of equipment and facilities for plant tissue culture is to arrange a visit to a plant tissue culture laboratory (de Fossard, 1976). Addresses of laboratories may be obtained from the national correspondent of the country's branch of the International Association for Plant Tissue Culture (IAPTC). The name and

Fig. 2.2 Orbital shaker for the aeration of liquid cultures. (Courtesy of Lab-Line Instruments.)



address of the national correspondent can be obtained from the IAPTC secretary. This position is currently held by Dr. J. M. Widholm, Department of Agronomy, University of Illinois, 1102 S. Goodwin, Urbana, IL 61801, U.S.A.

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3

Aseptic techniques

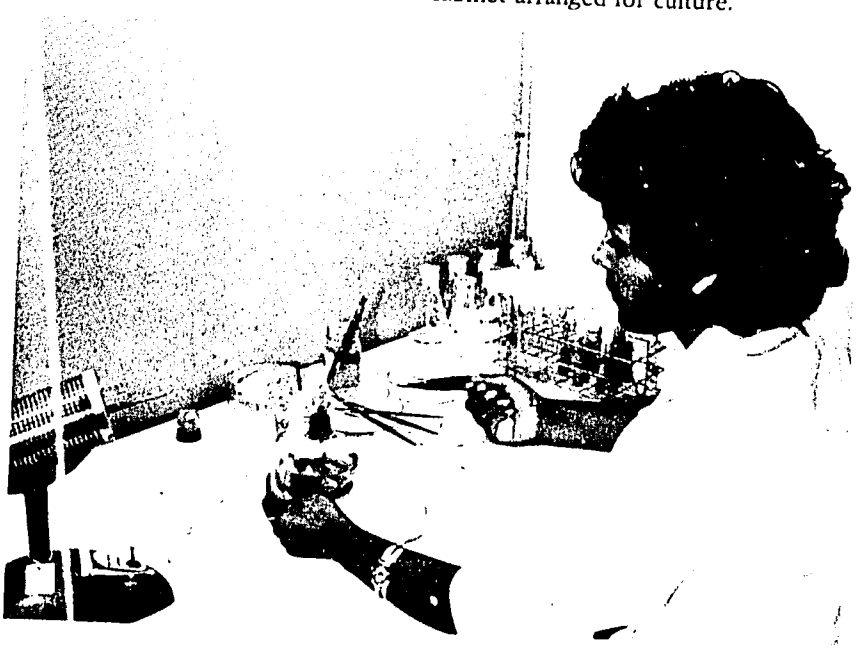
The importance of maintaining a sterile environment during the culture of plant tissues cannot be overemphasized. By following a few simple precautions to avoid microbial contamination, you will not waste valuable laboratory time in repeating experiments. The best example of the use of aseptic procedures is the operating room of a modern hospital, where the rigid precautions taken by surgeons are familiar to all of us.

The single most important factor in the selection of a suitable working area is the flow of air currents over the sterile area. Such air currents must be avoided because they carry spores of contaminating microorganisms. An interior room, similar to a photographic darkroom, is an excellent location for aseptic procedures. Because opening the door creates a draft, post a No Admittance sign on the door during the aseptic operations. If precautions are observed in a draft-free room, the open laboratory bench can be used. White (1963) mounted a transparent plastic shield parallel to the top of an open bench to prevent spore fallout. It is preferable, however, to conduct the aseptic procedures in some type of enclosed transfer chamber, bacteriological glove box, or laminar airflow cabinet (Fig. 3.1). Such enclosures are often equipped with a germicidal lamp emitting ultraviolet irradiation for the control of airborne contamination and for surface sterilization of the interior of the chamber. The emission at 253.7 nm is slowly germicidal, but it does not penetrate surfaces; dust and shadowed areas protect contaminants from its activity. Although these lamps are widely used, their effectiveness in creating a sterile environment is questionable (Klein and Klein, 1970; Collins and Lyne, 1984). UV lamps have a relatively short life, although they continue to emit visible light after emission at 253.7 nm has ceased. They should not be used in the presence of plastic apparatus, and the irradiation may produce inhibitory substances in culture media (Collins and Lyne, 1984; see Health hazards). The use of germicidal lamps should be kept at a minimum, since they are not a substitute for cleanliness. Laminar

flow cabinets are designed to direct a gentle flow of ultrafiltered sterile air across the working area, thus minimizing airborne contamination. Flaming instruments in such a facility should be done with caution. Alcohol is highly inflammable. The air stream from the cabinet would direct the flash fire toward the worker (Wetherell, 1982). After dipping in ethanol or isopropanol (80% v/v), instruments should be blotted dry on sterile filter paper. Before the start of any sterile procedure, the working area should be thoroughly scrubbed with a tissue soaked with ethanol or isopropanol (80% v/v).

Another extremely important point about aseptic procedure, and one of the leading causes of contamination, is dirty hands. Simply rinsing the hands with water is insufficient; it is essential that they be vigorously scrubbed with plenty of soap and hot water for several minutes. Attention must also be given to the fingernails and to any part of the forearm that extends into the chamber. Ideally, wash basins should have a foot- or elbow-operated mixer tap equipped with a spray nozzle (Collins and Lyne, 1984). After a hot water rinse, blot the skin partially dry with paper towels. It is unnecessary to use any strong disinfectants that might produce a skin rash. The hands may be sprayed with a dilute solution of ethanol or isopropanol, although this practice causes skin dryness and

Fig. 3.1. Laminar air-flow cabinet arranged for culture.



these mixtures must not be used indiscriminately around an open flame. According to Biondi and Thorpe (1981) the seed coat can be removed from sterile seeds manually without contamination by dipping the fingers repeatedly in ethanol (40–70% v/v) prior to the operation. Some years ago hexachlorophene (2,2′-methylenebis [3,4,6-trichlorophenol]) was widely used as an antibacterial agent in soaps. This chemical penetrates the intact skin, and it has the potential to cause brain lesions and other serious human disorders. For this reason it is not recommended that pHisohex or other hand-washing preparations containing hexachlorophene be used (*Science 175*, 148, 1972).

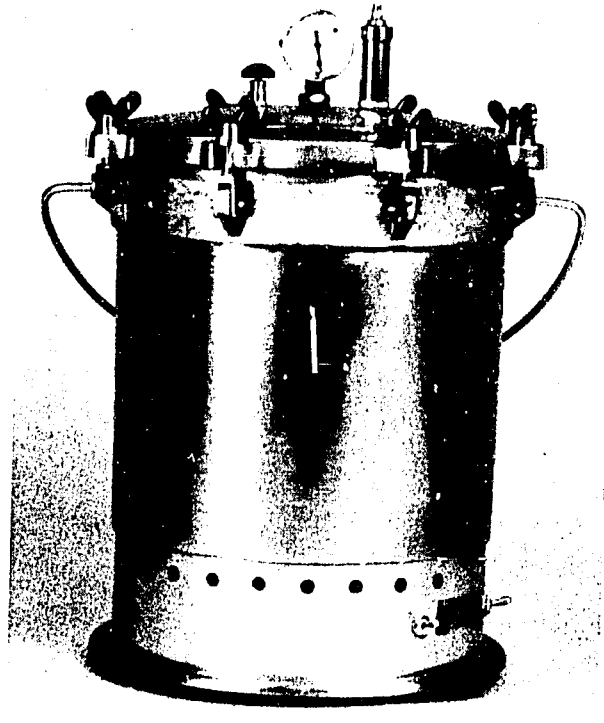
Several techniques are employed for the sterilization of glassware, surgical instruments, liquids, and plant material. The methods can be classified as follows: dry heat, wet heat, ultrafiltration, and chemical.

Dry heat. This method is used for glassware, metal instruments, or other materials that are not charred by high temperatures. Objects containing cotton, paper, or plastic cannot be sterilized with dry heat. Surgical blades and scalpels should not be sterilized by this method because the high temperature will dull the cutting edge. Although laboratory drying ovens may be used, the oven of a gas or electric stove will serve the same purpose. In calculating the time required for hot-air sterilization, three time periods must be considered. Approximately 1 hr (heating-up period) is allowed for the entire load to reach the sterilization temperature. The recommended holding periods at different sterilization temperatures are 45 min for 160°C, 18 min for 170°C, 7.5 min for 180°C, and 1.5 min for 190°C (Collins and Lyne, 1984). For a moderate oven setting of 160°C (320°F) a sterilization time of approximately 2 hr should be allowed. A cooling-down period is advisable in order to prevent the glassware from cracking due to a rapid drop in temperature. The objects to be sterilized are carefully wrapped in heavy-duty aluminum foil before being placed in the oven. In the United States three grades of aluminum foil are commercially available, and the heavy-duty grade is always used in tissue culture procedures. The thinner grade frequently has pinholes, and the extra-heavy-duty is too thick for wrapping objects. After sterilization the wrapped objects are taken to the transfer chamber.

Wet heat. This procedure employs an autoclave operated with steam under pressure (Fig. 3.2). If the laboratory is not equipped with an autoclave, a home pressure cooker can be used. For the sterilization of paper products, glassware, instruments, and liquid volumes not exceed-

ing 50 cm^3 per container, a steam pressure of 15 lb/in^2 (103.4 kPa) at a temperature of 121°C (250°F) is applied for 15 min. The minimum sterilization time increases with an increase in liquid volume. For example, with the preceding conditions of pressure and temperature, 75 cm^3 of liquid per container requires 20 min, 250–500 cm^3 requires 25 min, and a 30-min period is necessary for 1,000 cm^3 (Biondi and Thorpe, 1981). Do not start timing the sterilization period until the autoclave or pressure cooker has reached the proper temperature and all the residual air in the chamber has been displaced by steam. If a pressure cooker is used, do not close the escape valve until a steady stream of pure steam is evident. At the end of the sterilization period, the pressure must be permitted to return slowly to the atmospheric level because rapid decompression will cause the liquids to boil out of the vessels. Prolonged autoclaving must be avoided, because it results in the decomposition of the chemicals present in the medium. This topic will be discussed in Chapter 4. After autoclaving any paper products, the objects should be

Fig. 3.2. Portable electric autoclave for wet sterilization of media and equipment. (Courtesy of Gallenkamp.)



placed in the drying oven (<60°C) briefly in order to evaporate the condensed moisture. Steam in the autoclave chamber must penetrate the materials; a temperature of 121°C will not by itself achieve sterilization. With the exception of the flasks, instruments and other materials should be wrapped in heavy unwaxed paper. Although aluminum foil is commonly used as a wrapping, it is impermeable to the steam vapors and therefore is not recommended (Hamilton, 1973). Demineralized water should be used in boilers of autoclaves that generate their own supply of steam, as well as in pressure cookers. Steam generated by external power plants often is contaminated with various volatiles that may be absorbed by the objects within the autoclave (Bonga, 1982). Caution should be exercised in subjecting plastic labware to moist-heat sterilization (Biondi and Thorpe, 1981).

Another form of wet-heat sterilization is a boiling-water bath. Articles are placed in boiling water containing sodium carbonate (2% w/v) for 20 min. All vegetative microorganisms will be destroyed, although a few highly resistant spores may survive the treatment (Hamilton, 1973; Collins and Lyne, 1984).

Ultrafiltration. Some media components are unstable at high temperatures and must be sterilized by ultrafiltration at room temperature. Usually a small volume is sterilized by passage through a membrane filtration unit attached to a graduated syringe. For example, the Swinney and Swinnex are reusable devices equipped with membrane filters, whereas the Millex filter units are disposable (Millipore Corporation). The appropriate volume of the sterile liquid is added directly to the autoclaved medium. If an agar medium is employed, this is done while the agar is still warm and in the sol state. For large volumes, a larger filter holder is required. These units are equipped for either vacuum or pressure operation. Although several different kinds of ultrafilters are available, their various merits and disadvantages are beyond the scope of this introduction. The Nuclepore filter is made of polyethylene film punctured with holes of a given diameter. Most of the disposable membrane filters are composed of overlapping fibers of various cellulose esters that form a screen or sieve. The sterile filtration of nonaqueous solutions, e.g., containing the solvent dimethyl sulfoxide, requires a Fluoropore (Millipore) type filter of thin Teflon. Membrane filters are frequently impregnated with soluble surfactants. The release of these compounds by such filters has been shown to adversely affect the growth of cell cultures (Cahn, 1967). Prior to use the surfactant should be leached from the filter with hot water. Extract with approximately 200 cm^3 of hot (90–

100°C) DDH_2O. Discard the filtrate, attach another sterile flask to the filtration unit, and chill the apparatus in a refrigerator. Ultrafiltration at low temperature will extract a minimum of residual surfactant from the membrane filter. The Durapore (Millipore) membrane filter is made from a new fluorocarbon polymer that contains no surfactants and is recommended for the preparation of tissue culture media. Although a 0.4-μm filter is recommended for some procedures, the pore diameter should measure 0.22 μm for the complete removal of all bacteria and eukaryotic microorganisms. The membranes are delicate and should be handled only with blunt and flat forceps. A helpful guide on ultrafiltration is available from the Millipore Corporation.

Chemical sterilization. The working area is generally surface sterilized with either ethanol or isopropanol (70% v/v). Although acidified alcohol (70% v/v, pH 2.0) may be more effective as a disinfectant, it is not commonly used because of its corrosive effect on metal instruments. A slightly higher concentration of ethanol (80% v/v), a considerably more inflammable mixture, is employed for periodically sterilizing the instruments. An ethanol dip can be assembled by inserting a large test tube (25 mm O.D. × 150 mm) filled with ethanol (80% v/v) into the mouth of an empty metal can. After immersion in the alcohol, the instrument is then passed through the flame of a methanol lamp. Avoid prolonged heating of the instrument after evaporation of the alcohol. When not in use, the tube of ethanol should be capped to prevent evaporation. (See Health hazards.)

The surface sterilization of plant material may be accomplished with an aqueous solution of either sodium hypochlorite (NaOCl) or calcium hypochlorite ($Ca[OCl]_2$). Most laboratories use a household bleach such as Clorox. These commercial products usually contain 5.25% NaOCl as the active agent. When diluted with water (1 part bleach:9 parts water), the final sterilizing solution should contain not less than 0.5% NaOCl. Because of complete dissociation hypochlorite has relatively little activity at pH over 8.0, and it is much more effective by buffering the solution at about pH 6.0 (Behagel, 1971). Freshly excised pith or tuber tissue, completely immersed in the hypochlorite solution, will be surface sterilized after approximately a 10-min exposure. Following the hypochlorite treatment the plant material must be thoroughly rinsed with several changes of sterile DDH_2O in order to remove all traces of the disinfectant. Relatively large pieces of tuber tissue should be successively rinsed in three 600-cm^3 beakers, each beaker containing about 200 cm^3

of sterile DDH_2O. Because of the corrosive effect on metal instruments within the chamber, the hypochlorite solution and the rinse water should be discarded immediately after use.

Several agents were tested for the surface decontamination of seeds by Sweet and Bolton (1979). Calcium hypochlorite was found to be one of the most effective and least injurious agents. Sodium ions (i.e., in sodium hypochlorite) may induce abnormal development in some seedlings. In addition to $Ca(OCl)_2$ the mixture used by Sweet and Bolton contained a phosphate buffer, giving a final pH of 6.0, and a 1% (v/v) solution of either Triton or Tween-80 as a wetting agent. The seeds were immersed in the disinfecting solution for 10 min and then rinsed three times with sterile water.

Some workers prefer a two-stage disinfection of seed surfaces, although the possible advantage of this approach has not been scientifically determined. The seeds are first immersed in ethanol (70% v/v) and agitated for 1 or 2 min, and then transferred aseptically to a second vessel containing the hypochlorite solution for approximately 20–25 min. Alcohol alone has also been used as a surface sterilant in some studies on woody plants (Bonga, 1982). The surface sterilization of plant parts containing either cutin, suberin, or epidermal appendages requires the addition of a small amount of detergent (0.05% v/v) like Teepol or Tween-80 to the hypochlorite solution (Street, 1973).

Some plant tissues pose a special problem if they contain microorganisms within the tissue sample. Surface sterilization is obviously ineffective. If a fleshy organ selected for explant preparation shows any localized internal discoloration, it should be discarded. The goal in surface sterilization is to remove all of the microorganisms with a minimum of damage to the plant system to be cultured. In some cases, the achievement of this goal is empirical and the worker must be flexible in the approach (de Fossard, 1976). In the case of seeds, the use of higher concentrations or longer periods of treatment does not appear to improve the decontamination success without reducing the percentage of germination (Sweet and Bolton, 1979).

This does not exhaust the list of possible chemical sterilants, and there are some that are not recommended for the plant tissue culture laboratory. These will be discussed later in this chapter in the section on health hazards.

Antibiotics. Although antibiotics are employed routinely in animal cell cultures, they have not been widely used in plant tissue cultures (see

Eichholtz, Hasegawa, and Robitaille, 1982). The early botanists were aware that these natural products may alter the growth and development of plant tissues cultured in vitro (Gautheret, 1959; Butenko, 1964). In our opinion this practice should be avoided, as it is not a substitute for the strict adherence to proper sterile techniques. In fact, no known antibiotic is effective against all microorganisms that might cause contamination. These antibiotics, or their degradation products, may be metabolized by plant tissues with unpredictable results. Xylogenesis in explants of lettuce pith and Jerusalem antichoke tuber was strongly inhibited in the presence of gentamicin sulfate, although this effect was observed within the concentration range (50–100 $\mu g/cm^3$) recommended by the manufacturer for use in tissue cultures (Dodds and Roberts, 1981). This antibiotic was also found to be inhibitory to tobacco callus growth and shoot initiation from tobacco callus and *Salpiglossis* leaf discs (Eichholtz et al., 1982).

High-temperature degradation of media components. Several chemicals employed in plant tissue culture media degrade on exposure to steam sterilization. Gibberellins are rapidly degraded by elevated temperatures, and the biological activity of a freshly prepared solution of GA_3 was reduced by more than 90% as a result of autoclaving (Bragt and Pierik, 1971). The auxins NAA, IAA, and 2,4-D are thermostabie. Although IAA is unaffected by autoclaving under normal conditions, it is subject to decomposition by other factors found under in vitro conditions (Posthumus, 1971; Yamakawa et al., 1979). Aqueous solutions of kinetin, zeatin, and IPA have been chromatographed on thin-layer silica-gel chromatograms before and after prolonged autoclaving with no breakdown products detected (Dekhuijzen, 1971). On the other hand, biologically inactive 1,3- or 9-substituted purine molecules were converted into callus-inducing N^6-substituted purines by autoclave treatment. Crude plant extracts that possibly contain nonactive purine molecules should be filter sterilized. Although heat sterilization apparently has no effect on the isomers of abscisic acid, this hormone is sensitive to light (Wilmar and Doornbos, 1971). Vitamins have varying degrees of stability. In general, the addition of vitamins to culture media prior to autoclaving is not advisable; filter sterilization at room temperature is preferable. Thiamine-HCl can be heated to 110°C in an aqueous solution without decomposition, but if the pH of the solution is above 5.5, it is rapidly destroyed (Windholz, 1983). Calcium pantothenate cannot be autoclaved without destruction, whereas pyridoxine-HCl is thermostable. One of the most frequently employed carbohydrates in media is sucrose. This

sugar decomposes, to some extent, on autoclaving to release a mixture of D-glucose and D-fructose (Ball, 1953), and these monosaccharides are apparently inhibitory to some cultured tissues (Stehsel and Caplin, 1969; Wright and Northcote, 1972). A discussion of the use of autoclaved sugars in culture media was given by Roberts (1976). Steam sterilization may also catalyze reactions within the media between carbohydrates and amino acids (Peer, 1971).

Health hazards. Hypochlorite solutions should be used with care. Inhalation can produce severe bronchial irritation and skin contact can be harmful (Windholz, 1983). One should never use the mouth to pipette a hypochlorite solution. Pipette fillers are safe and relatively inexpensive. Never use hypochlorite or other inorganic chloride preparations in the presence of UV irradiation. The resulting release of free chlorine gas is a serious health hazard (Hamilton, 1973).

A fire danger exists if the student, after flaming an instrument, reinserts the hot instrument into the alcohol dip. Ethanol is highly inflammable, and one must be extremely careful about spilling ethanol or other alcohol in the vicinity of an open flame. The vessel of alcohol (dip) should be kept in a metal can to avoid the release of burning alcohol in case of glass breakage.

UV irradiation poses some serious health risks. One should never look at a live tube with the naked eye. UV burns to the eye (actinic keratitis) are very painful, although not normally of lasting effect (Scherberger, 1977). A glass barrier, between the eyes and the UV source, provides complete protection. Also, UV irradiation can produce irritation to unprotected skin; so avoid placing the hands in the transfer chamber when the lamp is on. Another problem is the formation of ozone (O_3) resulting from the photochemical reaction with atmospheric oxygen. This explosive gas is a powerful oxidizing agent, and high concentrations can cause severe irritation to the respiratory tract and eyes. Symptoms of ozone toxicity have been experienced by airline passengers and crew during high-altitude flights (*Science 205*, 767, 1979). The UV lamp should never be left on for long periods of time with the transfer chamber sealed.

Mercuric chloride ($HgCl_2$) has been used as a disinfectant, although this chemical is an extremely dangerous poison. A solution of mercuric chloride is slightly volatile at room temperature, and this has resulted in cases of mercury poisoning to laboratory workers.

Although Lysol (3.5% v/v) kills vegetative cells, it is ineffective in eliminating spore contamination. Lysol is a preparation of cresols and phenol and consequently leaves an oily film on surfaces. In addition, it

will produce a severe burn to the skin. If residual Lysol is autoclaved it will result in the chemical contamination of the contents of the au toclave plus the autoclave itself (Hamilton, 1973). Because Lysol is a common constituent of industrial floor cleaners, one should make certain that such agents are not used in the tissue culture laboratory. The volatile phenols can adversely affect the cultures (Hamilton, 1973).

Gas sterilization should not be used in a classroom laboratory without strict supervision. Small plastic objects can be sterilized by subjecting them to a saturated atmosphere of ethylene oxide for several hours in a sealed container at room temperature. It is violently explosive in nearly all mixtures with air and is toxic at concentrations not detected by smell (Sykes, 1969). The gas is highly irritating to the eyes and mucous membranes, and high concentrations can cause pulmonary edema. Basic procedures for its use may be found in the review by Hamilton (1973). In addition, the sterilization of polycarbonate culture flasks with ethylene oxide can result in the formation of some substances that are mutagenic to cultured animal cells (Krell, Jacobson, and Selby, 1979).

Avoiding contamination. One of the most important factors in preventing contamination is the prevention of airborne microorganisms from reaching the sterile area. Always keep all windows and doors closed in order to avoid drafts. A minimum number of persons should be present in the vicinity of the transfer chamber. Avoid breathing into the transfer chamber. A surgical face mask is often worn by those working in laminar flow cabinets. Keep your hands, wrists, and arms scrupulously clean, and never pass your hand or arm over a sterile exposed surface (e.g., a water rinse or an open agar plate). All sterile open surfaces should be placed as far back in the transfer chamber as conveniently possible. When pouring sterile liquids, grasp the flask at the base, and keep the hands as far as possible from the open tube or Petri dish receiving the liquid. In opening a sterile Petri dish, hold the lid with the thumb and middle finger on opposite sides, and gently pull the lid back. That is, never permit the fingertips to pass over the sterile bottom half of the dish. A common practice in microbiology is to flame the mouth of culture tubes and flasks during aseptic procedures. There is the possibility of introducing high concentrations of ethylene into the vessels by this practice. This gas, a product of combustion processes, is a plant hormone and can have profound effects on the growth and morphogenesis of plant tissue cultures (Beasley and Eaks, 1979). At the conclusion of each step of the procedure, remove all unnecessary glassware, instruments, aluminum foil, and

other materials that have been used. Finally, it is very important to remove all contaminated cultures from incubators and plant growth chambers. Discarded cultures should never be permitted to accumulate in the laboratory. They will produce spores and place the entire operation in jeopardy.

QUESTIONS FOR DISCUSSION

1. Can you think of methods of avoiding contamination that were not mentioned in this chapter?
2. What are some potential health hazards associated with plant tissue culture procedures?
3. What are some of the chemical disinfectants that may be considered dangerous to your health?
4. What media components are most easily degraded or chemically altered by the high temperatures and pressures associated with steam sterilization?

APPENDIX

Flasks containing double-distilled water and culture media can be sealed for sterilization with a single layer of heavy-duty aluminum foil. Center the square of foil over the mouth of the flask. Make a circle by pressing the tip of the thumb and index finger together, and with a downward movement press the foil firmly against the neck of the flask. The foil should extend down at least 20–30 mm from the mouth of the flask. The foil cap, however, should not be airtight, or it could be blown off during autoclaving by the expansion of the trapped air within the vessel. In addition, it is necessary that the steam come into contact with the medium in order to achieve sterilization.

After placing the explants on the surface of the medium, some research workers seal the culture tubes with one or more layers of Parafilm M. This material is placed on top of the previously sterilized aluminum foil cap. The sealing agent is a rubber-wax-polymer mixture approximately 120 μm in thickness. The manufacturer, American Can Company, has informed us that the membrane will act as an ultrafilter for gas diffusion (i.e., it will impede the flow of gases in and out of culture vessels). The concentration of ethylene produced by cultured tissues may rise to toxic levels in vessels sealed with Parafilm M. In fact, the production of ethylene from the combustion of a methanol lamp may influence cultured plant tissues (Beasley and Eaks, 1979).

As an alternative to aluminum foil, polypropylene film has been used as a capping material for tissue culture containers (Mahlberg, Masi, and Paul, 1980). This transparent thin film, 1 mil or less in thickness, offers

certain advantages. Although the film is relatively impermeable to water vapor and prevents desiccation of long-term cultures, gas exchange is not restricted. The transparency of the film is an advantage for cultures requiring illumination. The film is cut into squares of an appropriate size, and individual pieces are placed between the pages of a booklet made from ink-free paper. The booklet containing the film is enclosed in an envelope for sterilization by autoclave. Culture tubes, capped in the conventional manner with aluminum foil, are oven sterilized and filled with sterile nutrient medium. The sterile polypropylene is then used to seal the tubes after the explants have been placed on the medium, and the foil caps are discarded. The film is held in position with small rubber bands. The plastic caps are discarded at the termination of the experiment (Mahlberg et al., 1980). Although polypropylene film is widely available, it can be ordered directly from Polypropylene Films Corporation, 1571 Timber Court, Elgin, IL 60120 (specify uncoated, type 07 or 08, 1 mil in thickness).

The use of sterile disposable equipment is a time saver in a busy laboratory. Disposable pipettes can be used for dispensing filter-sterilized fluids. Plastic sterile Petri dishes are manufactured in several sizes, and cluster dishes are useful when several treatments are involved in an experiment.

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4

Nutritional components of tissue culture media

Because there is a division of labor by the different organs of the plant in the biosynthesis of essential organic metabolites, we know much less about the nutritional requirements of the various organs and tissues of the plant than we do of the whole plant. A tomato plant growing in the garden requires only an external supply of mineral elements for the successful completion of its life cycle. An excised root of this tomato plant does not have precisely the same requirements for normal growth and development. In addition to the essential minerals, the excised root also needs the addition of certain organic compounds to the culture medium because in the whole plant the roots were provided with these compounds, which were synthesized elsewhere and transported into the root system. In 1934 White discovered that isolated tomato roots had the potential for unlimited growth, if they were provided with a liquid medium containing a mixture of inorganic salts, sucrose, thiamine, pyridoxine, nicotinic acid, and glycine. In addition to the somewhat selective biosynthetic activity of certain isolated tissues and organs, cultured systems may exhibit changes in their metabolic pathways over a period of time. These changes in metabolism require corresponding changes in nutritional requirements. The requirement for a particular organic supplement could be due either to the inability of the cultured tissue to produce it or to a new requirement resulting from a shift in metabolism.

The components of plant tissue culture media discussed in this chapter include inorganic salts, plant growth regulators, vitamins, amino acids and amides, complex organic supplements, charcoal, carbon sources, osmotica, water, and the medium matrix.

Inorganic salts. Cultured plant tissues require a continuous supply of certain inorganic chemicals. Aside from carbon, hydrogen, and oxygen, the essential elements required in relatively large amounts are termed macronutrient elements and these include nitrogen, phosphorus, potas-

sium, calcium, magnesium, and sulfur. Nitrogen, added in the large amount, is present as either a nitrate or ammonium ion, or a combinatio of these ions. Magnesium sulfate ($MgSO_4 \cdot 7H_2O$) satisfies both the ma nesium and sulfur requirements. Phosphorus can be represented l either $NaH_2PO_4 \cdot H_2O$ or KH_2PO_4. Potassium, the cation found in th largest amount, is given as either KCl, KNO_3, or KH_2PO_4. Eith $CaCl_2 \cdot 2H_2O$, $Ca(NO_3)_2 \cdot 4H_2O$, or anhydrous forms of either salt, ca be added for the calcium requirement.

In addition to the macronutrient elements, plant cells require trac of certain micronutrients. Because these quantities are exceedingly sma for some of the elements, a concentrated stock solution is prepared i advance.[1] Micronutrient elements required by all higher plant cells in clude iron, manganese, zinc, boron, copper, molybdenum, and chlorin Although sodium is not generally required by higher plants, this elemer may be a micronutrient for cultures of halophyte tissues, plants with C photosynthetic pathways, and plants with Crassulacean acid metabolisr (CAM). An iron stock solution is prepared separately because of th problem of iron solubility. Usually the iron stock is prepared in a chelate form as the sodium salt of ferric ethylenediamine tetra-acetate (Na FeEDTA). In addition to the known micronutrients, some media contai traces of cobalt and iodine. The possible essentiality of nickel, titanium beryllium, and aluminum is questionable. One unresolved problem i that even the purest chemical reagents contain traces of inorganic con taminants, and these elements constitute a hidden source of micro nutrients (Yeoman, 1973). Agar is also a source of numerous minera elements. Unfortunately, little critical work has been done on the micronutrient requirements of callus cultures.

Plant growth regulators. The growth regulator requirements for most callus cultures are auxin and cytokinin (Fig. 4.1).[2] Auxins, a class of compounds that stimulate shoot cell elongation, resemble IAA in their spectrum of activity. Cytokinins, which promote cell division in plant tissues under cetain bioassay conditions, regulate growth and development in the same manner as kinetin (6-furfurylaminopurine). Cytokinins are mainly N^6-substituted aminopurine derivatives, although there are some

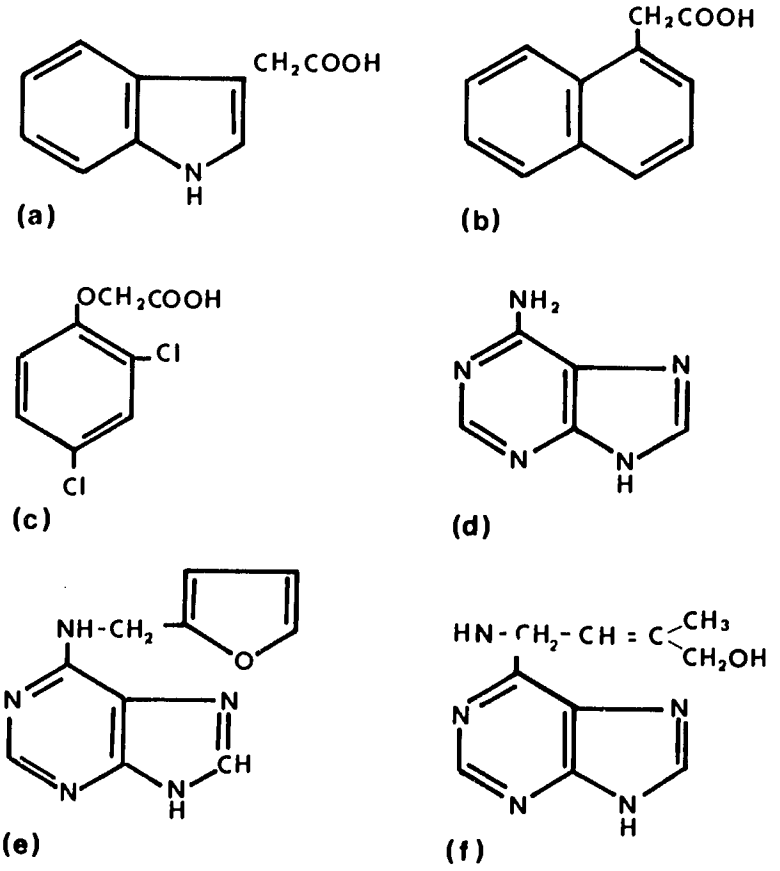
[1] It is also convenient to prepare in advance stock solutions of macronutrients, vitamins, and certain plant growth regulators. These solutions can be stored for limited periods of time in glass containers at 4°C.

[2] The term "hormone" should be reserved for naturally occurring plant growth regulators. Although α-NAA, 2,4-D, and kinetin are plant growth regulators, they are not considered to be plant hormones.

exceptions. Auxin–cytokinin supplements are instrumental in the regulation of cell division, cell elongation, cell differentiation, and organ formation. Gibberellins are rarely added to culture media, although GA_3 has been used in apical meristem cultures (Morel and Muller, 1964). Increasing attention has been given to ethylene in the initiation of buds (Thorpe, 1982b) and tracheary element differentiation (Miller and Roberts, 1984). Relatively few studies have employed abscisic acid as a supplement to tissue culture media.

The auxins most frequently employed are IAA, α-NAA, and 2,4-D.[3]

Fig. 4.1. Structural formulae of some auxins and cytokinins. Auxins include (a) indole-3yl-acetic acid, (b) α-naphthaleneacetic acid, (c) 2,4-dichlorophenoxyacetic acid. Cytokinin activity is shown by (d) adenine, (e) kinetin, (e) and (f) *trans*-zeatin.



[3] Both the α and β isomers of NAA are commercially available, but the α isomer is always used in culture media. The β isomer is a weak auxin with relatively little physiological activity.

Callus formation in cereal cultures is stimulated, however, by 2,4,5-T. Indole-3-butyric acid (IBA) and *p*-chlorophenoxyacetic acid (4-CPA) are also effective auxins. IBA is a particularly effective rooting agent. IAA is a naturally occurring auxin, but unfortunately it is readily degraded by light and enzymatic oxidation. Because IAA oxidase may be present in cultured tissues, IAA is added to media in relatively high concentrations (1–30 mg/l). Since α-NAA is a synthetic chemical, it is not subject to the same enzymatic oxidation as IAA. It may be added in lower concentrations (0.1–2.0 mg/l). The most effective auxin for callus proliferation for most cultures is 2,4-D (10^{-7}–10^{-5}M), often in the absence of any exogenous cytokinin. This herbicide is a powerful suppressant of organogenesis and should not be used in experiments involving root and shoot initiation (Gamborg et al., 1976).

There are several exceptions to this dual requirement for auxin and cytokinin. Some cultures require no exogenous auxin in the medium (Street, 1966). Although some tissue explants initially may have high endogenous auxin levels, the cultured tissues apparently develop auxin biosynthetic pathways. The terms "habituation" and "anergy" have been given to this autonomous condition, originally studied in cultures of tumor tissue from diseased plants. Other cultures have been found that require the addition of auxin but not cytokinin. The heritable conversion of cultured tobacco cells to the cytokinin-habituated phenotype occurs in response to cytokinin or high temperatures (Meins and Lutz, 1980). Although the habituated state is highly stable, reversion occurs when cloned lines are induced to form plants (Meins and Binns, 1982).

The most widely used cytokinins in culture media are kinetin, benzyladenine, and zeatin. Kinetin and benzyladenine are synthetic compounds, whereas zeatin occurs naturally. Another naturally occurring cytokinin, which is considerably less expensive than zeatin, is 6-[γ, γ-dimethylallylamino] purine or N^6-[Δ^2-isopentyl]-adenine. Diphenylurea, a growth factor reported present in coconut milk (see Jacobs, 1979), exhibits cytokininlike responses in some bioassays. Kinetin is typically added at a concentration of 0.1 mg/l for the induction of callus. A preparation of autoclaved coconut milk can be added to culture media as a cytokinin source for a final concentration in the medium of 10–15% v/v.

Vitamins. Vitamins have catalytic functions in enzyme systems and are required only in trace amounts. Some consider that thiamine (vitamin B_1) may be the only essential vitamin for nearly all plant tissue cultures,

whereas nicotinic acid (niacin) and pyridoxine (vitamin B_6) may stimulate growth (Gamborg et al., 1976; Ohira, Ikeda, and Ojima, 1976). Thiamine is added as thiamine-HCl in amounts varying from approximately 0.1 to 30 mg/l. The need for thiamine by tissue cultures is especially evident at low levels of cytokinins. In the presence of fairly high concentrations of cytokinin (0.1–10 mg/l), tobacco cells grow without the addition of exogenous thiamine (Digby and Skoog, 1966; Linsmaier-Bednar and Skoog, 1967). These tobacco cultures apparently develop the capability of synthesizing thiamine (Dravnicks, Skoog, and Burris, 1969).

Both nicotinic acid and pyridoxine have been reported to be required for the culture of *Haplopappus gracilis* (Eriksson, 1965).

Some other vitamins that have been used in plant tissue culture media include *p*-aminobenzoic acid (PABA; vitamin B_x), ascorbic acid (vitamin C), biotin (vitamin H), choline chloride, cyanocobalamin (vitamin B_{12}), folic acid (vitamin Bc), calcium pantothenate, and riboflavin (vitamin B_2) (Huang and Murashige, 1977; Gamborg and Shyluk, 1981).

Ascorbic acid, which may be employed with other organic acids, is useful as an antioxidant to alleviate tissue browning (Reynolds and Murashige, 1979).

As indicated in Chapter 3, some of the vitamins are heat labile. Therefore it is always advisable to filter sterilize vitamin preparations and add them by pipette or graduated syringe to the warm unsolidified autoclaved medium.

Amino acids and amides. With the exception of glycine (aminoacetic acid), which is a component of several media, amino acids are not usually added to plant culture media. If a mixture of organic nitrogen is considered necessary, the medium can be enriched with either casein hydrolysate or casamino acids (0.05–0.1% w/v). Hydrolysis of the milk-protein casein can be achieved by different methods, and the hydrolysate consists of an ill-defined mixture of at least 18 different amino acids (Klein and Klein, 1970). If the addition of the hydrolysate results in a beneficial effect, additional experiments should be made substituting various amino acid and amide mixtures for the hydrolysate. Ultimately the specific organic nitrogen requirements can be identified. The composition of different mixtures of amino acids and amides that have been used in plant culture media is given by Huang and Murashiage (1977). Some of the amino acids or amides that most frequently produce positive results are L-aspartic acid, L-asparagine, L-glutamic acid, L-glutamine, and L-arginine. Traces of L-methionine added to the medium for the enhancement

of ethylene biosynthesis have a stimulatory effect on xylogenesis (Roberts and Baba, 1978; Miller and Roberts, 1984). It has not been established that supplements of nucleotides are necessary for plant tissue cultures (Gamborg and Shyluk, 1981). It is not uncommon to observe growth inhibition following the addition of a combination of amino acids, a phenomenon that has been attributed to competitive interactions between the various amino acids (Street, 1969).

Complex organic supplements. The trend in plant tissue culture has been to attempt to define the constituents of a given medium and eliminate the use of crude natural extracts. Such products as peptone, yeast extract, and malt extract are used infrequently today. Although this attitude is commendable from a scientific viewpoint, the use of natural extracts should not be ignored when chemically defined mixtures fail to produce the desired results. For this reason, a procedure for the preparation of coconut milk from fresh coconuts has been included in the appendix. Fruit juices are also important organic supplements. Einset (1978) found the in vitro growth of explants from several *Citrus* sp. was greatly stimulated by the addition of orange juice to the medium. Tomato juice (30% v/v) has also been used effectively under certain conditions (Nitsch and Nitsch, 1955; Straus, 1960). Orchid media has been supplemented with banana fruit extract (Arditti, 1968) and fish emulsion (Withner, 1959).

Charcoal. Activated charcoal will adsorb many organic and inorganic molecules from a culture medium (Mattson and Mark, 1971), and this substance has been used in a variety of tissue culture systems. Although the precise effects of activated charcoal are unknown, there are several possible modes of operation. It may remove contaminants from agar (Kohlenbach and Wernicke, 1978) and secondary products secreted by the cultured tissues (Wang and Huang, 1976; Fridborg et al., 1978), or possibly regulate the supply of certain endogenous growth regulators (Reinert and Bajaj, 1977). In addition, some of the effects of activated charcoal may be due to darkening of the support matrix and thus approximating more closely soil conditions (Proskauer and Berman, 1970). As a medium supplement activated charcoal has been reported to stimulate embryogenesis (Kohlenbach and Wernicke, 1978). On the other hand, the presence of this adsorbent can have inhibitory effects on growth and morphogenesis in vitro (Constantin, Henke, and Mansur, 1977; Fridborg et al., 1978). The type of activated charcoal used is important, because the adsorptive characteristics are dependent on the man-

ufacturing process (Bonga, 1982). Wood charcoal is considerably higher in carbon content in comparison to bone charcoal, and the latter preparation contains ingredients that may adversely affect plant tissue cultures (Bonga, 1982). Further information on the effects of charcoal-supplemented media can be found in chapters 11 and 14.

Carbon sources. All media require the presence of a carbon and energy source. Sucrose or D-glucose is usually added in concentrations of 20,000–30,000 mg/l, although higher levels may be advantageous for some purposes. Nearly all cultures appear to give the optimum growth response in the presence of the disaccharide sucrose, whereas there can be considerable variability in growth when other disaccharides or monosaccharides are substituted for sucrose. Although many laboratories autoclave the sucrose with the remainder of the nutrient medium, sucrose is heat labile, and the result is a combination of sucrose, D-glucose, and D-fructose. Such an autoclaved medium may give completely different results compared to a medium containing filter-sterilized sucrose (Ball, 1953). The cyclitol *myo*-inositol is added to some culture media as a growth factor at a concentration of 100 mg/l. The choice and concentration of the sugar to be used depend mainly on the plant tissue to be cultured and the purpose of the experiment. For example, the induction of xylem differentiation can be greatly influenced by the type of carbohydrate employed in the medium (Roberts, 1976). In fact, xylogenesis can be induced by using either glycerol (2% w/v) or *myo*-inositol (2% w/v) as the principal exogenous carbon source (Roberts and Baba, 1982). The question of the purity of the carbohydrates has been raised because the occlusion of a variety of organic substances, particularly traces of amino acids, occurs during the crystallization of sucrose (Schneider, Emmerich, and Akyar, 1975). Street (1969) summarized some of the tissue culture studies utilizing various carbohydrate sources.

Osmotica. The uptake of water by plant cells is governed by the relative water potential values between the vacuolar sap and the external medium. The major components of the nutrient medium that influence water availability are the concentrations of the agar, the carbon source, and any nonmetabolite added as an osmoticum. One colloidal characteristic of the gel state of agar is the imbibitional retention of water within the micelles of the gel (Levitt, 1974). Carbohydrates not only function as a carbon source in metabolism, but they also play an important role in the regulation of the external osmotic potential. Often a weakly metabolized

sugar, e.g., mannitol or sorbitol, is used as an external osmoticu (Brown, Leung, and Thorpe 1979; Brown and Thorpe 1980). Mannit however, is metabolized by *Fraxinus* cultures (Wolter and Skoog, 196 and possibly others. Polyethylene glycol (PEG) has been used as an o moticum in protoplast fusion experiments (see chap. 13) and in the cry preservation of cultures (see chap. 15). Some additional informatic on the use of osmotica can be found in the review by Bonga (1982).

Water. The water employed in all tissue culture media, including th water used during the culture procedure, should be double distilled c demineralized distilled. In either case, it is mandatory that glass disti lation be the final step. Water distillation is a complex process, and vo atile organics of low molecular weight often are distilled along with th water. Bonga (1982) suggests that the distilled water collected durin the first 10–15 min of still operation should be discarded in order t eliminate the early vaporization of some of these volatile organic mol ecules. The use of an ion exchange column poses some technical prob lems in water purification because of the release of a variety of organi contaminants, including some metabolic products secreted by micro organisms growing within the column (Bonga, 1982). One should be cau tioned against the prolonged storage of redistilled water in polyethylen containers since these receptacles release substances that may be toxi to the cultures (Robbins and Hervey, 1974). It is unwise to store double- distilled water in Pyrex vessels for prolonged periods because detectable amounts of bacteria may accumulate during storage under nonsterile conditions (Street, 1973). The lengthy storage of sterile water is unwise, and this has been a problem in some of our hospitals (Favero et al., 1971).

Medium matrix. Unless the culture is prepared in an aqueous medium, it is grown on a semisolid or solid matrix. Contaminants, released by the matrix, may contribute to the nutrition of the cultured tissue. Most stationary cultures are grown on an agar base, e.g., Difco Bacto Agar, in a concentration range from about 0.6 to 1.0% w/v. Although commercial agar preparations contain a wide variety of organic and inorganic contaminants, several companies now market tissue culture agars (see Commercial sources of supplies). The analyses given by the Difco Laboratories on the elemental composition of Bacto Agar, Noble Agar, and Purified Agar provide no information on the possible presence of toxic substances, growth stimulants, or vitamins (Pierik, 1971). Romberger and

Tabor (1971) reported that an "agar inhibitory effect" was largely removed by autoclaving the medium with sucrose. A starch copolymer has been used as an agar substitute (Cooke, 1977), and a sucrose polymer has served as a support matrix (Tran Thanh Van and Trinh, 1978). Some additional gelling agents are discussed by Klein and Klein (1970).

In addition to gels, other materials have been tried. Filter-paper platforms were introduced by Heller (1965), and filter-paper discs impregnated with nutritives have been used (Phillips and Dodds, 1977). Glass fiber filters are useful supports for cultures, although these filters should be pretreated to remove contaminants and to saturate the cation exchange sites on the glass fibers with specific ions (Tabor, 1981). An unusual material was used by Cheng and Voqui (1977) in the culture of Douglas fir plantlets. These workers employed a synthetic polyester fleece (Pellon Corporation). Filter paper has been used to separate contiguous cultures of two different origins, i.e., as a "nurse" culture. To measure the growth of a culture with a minimum of disturbance, a thin layer of cells can be separated from the agar medium by a filter-paper disc. The disc and the cultured cells can be periodically removed, weighed aseptically, and replaced on the medium without sacrificing the cells (Horsch, King, and Jones, 1980).

Suggestions on the selection of a medium. The choice of a particular medium depends mainly on the species of plant, the tissue or organ to be cultured, and the purpose of the experiment. If the plant material has been cultured successfully in other laboratories, it is best to start with published methods. A suitable starting point for the initiation of callus from a dicot tissue explant would be the preparation of the MS basal medium. One characteristic of this medium is its relatively high concentration of nitrate, potassium, and ammonium ions in comparison to other nutrient media. The B5 medium developed by Gamborg's group (Gamborg, Miller, and Ojima, 1968) is also an effective nutrient mixture. In addition to the basal mineral mixture it is recommended that the MS vitamin mixture, *myo*-inositol (100 mg/l), and sucrose (2–3% w/v) be used for callus initiation. A possible modification of the MS vitamin mixture would be to increase the thiamine content. Gamborg's B5 medium contains 10 mg/l thiamine in comparison to 0.1 mg/l thiamine in the MS medium. For callus formation the addition of 2,4-D (0.2–2.0 mg/l) is effective for many tissues. The addition of a cytokinin (0.5–2.0 mg/l) may be helpful. Another combination for the production of callus is 2,4-D plus coconut milk. If these combinations fail to produce the desired results, a sup-

plement of amino acids or some natural plant extract may be considered. Some media contain combinations of auxins. Monocot cultures respond to the Schenk and Hildebrandt (1972) medium, which contains a combination of 2,4-D, *p*-chlorophenoxyacetic acid, and kinetin. Callus induction in some cereal plants requires high levels of 2,4-D (5–10 mg/l), according to Yamada (1977). Monocot cultures also have responded favorably to the addition of such auxins as 2,4,5-T, dicamba, and benazolin (Green, 1978).

Aside from considerations of callus formation, the initiation of various morphogenetic events in vitro often requires special adjustments in the concentrations of the components of the medium. Embryogenesis responds favorably to high potassium levels in some systems (Brown, Wetherell, and Dougall, 1976). In addition to the proper ratio of hormones, shoot formation may require a medium either high in phosphate (Miller and Murashige, 1976), or low in ammonium nitrate (Pierik, 1976). The total salt concentration may be a factor of some importance (Bonga, 1982).

Several reviews are devoted to various aspects of the nutrition of plant tissue cultures (Ojima and Ohira, 1978; Dougall, 1980; Gamborg and Shyluk, 1981).

QUESTIONS FOR DISCUSSION

1. Prepare a list of inorganic chemicals that you consider suitable for the macronutrient requirements of a typical dicot callus culture. What are the approximate concentrations that you would employ in the preparation of the culture medium?
2. What are some of the sources of inorganic and organic contaminants that are unwittingly added to our cultures?
3. What evidence can be given to show that cultured plant tissues experience changes in certain biochemical pathways during culture?
4. What are some possible substitutes for agar as a medium matrix? Can you think of any substitutes not mentioned in this chapter? Discuss their possible advantages and disadvantages.

APPENDIX

Preparation of coconut milk

Ripe coconuts can be purchased from local fruit markets, in some cases having been dehusked. Market coconuts contain about 100–200 cm^3 of liquid endosperm. Three micropyles ("eyes") are located at one end of the coconut, one of which is composed of relatively soft tissue, easily

removed with a cork borer. The liquid will pour more rapidly if an air vent is created by penetrating a second micropyle with an electric drill or a hammer and nail. Each coconut should be drained separately because occasionally one may open a coconut with fermented milk. This milk, easily identified because of its odor and appearance, should be discarded. The collected milk is filtered through several layers of cheesecloth. Boil the filtrate for approximately 10 min in order to precipitate the proteins. Cool to room temperature, decant, and filter the supernatant through a fairly rapid qualitative filter paper. For the preparation of 1 liter of nutrient medium, add 100–150 cm^3 of coconut milk. Any unused coconut milk can be frozen for use at a later date. Melting and refreezing apparently do not diminish the cytokininlike properties of the substances present in the liquid endosperm (Klein and Klein, 1970). For additional information see J. L. Riopel (1973), *Experiments in developmental botany*, Dubuque, Iowa: Brown.

How to prepare stock solutions

Prior to the preparation of a stock solution, always ask yourself the following question. How much of the chemical must be added to the quantity of the medium required? Let us say that 250 cm^3 of a nutrient medium is required containing 0.1 mg/l kinetin (equals 0.01 mg/100 cm^3 or 0.025 mg/250 cm^3). Now consider a 1.0-cm^3 pipette containing a kinetin solution of 0.025 mg/cm^3. The latter concentration is equivalent to 0.25 mg/10 cm^3, 2.5 mg/100 cm^3, and 25 mg/1,000 cm^3. Choose a convenient amount of kinetin to weigh out, e.g., 2.5 mg, dissolve the chemical in a few drops of 1 N HCl, and bring the final volume to 100 cm^3 with DDH_2O in a volumetric flask.

Solvents for plant growth regulators

Often the choice of a proper solvent for a given plant growth regulator is perplexing. Cytokinins are readily soluble in 1 N HCl. The indole auxins and NAA can be dissolved in 1 N NaOH. Although 2,4-D is soluble in ethanol, the use of dimethyl sulfoxide (DMSO) is recommended. This powerful solvent has been used previously in several physiological studies (Schmitz and Skoog, 1970; Delmer, 1979; Roblin and Fleurat-Lessard, 1983). At high concentrations, however, DMSO may have adverse effects on metabolism (Vannini and Poli, 1983). Caution

should be exercised in the use of DMSO, since it readily penetrates the skin and may have toxic effects (Windholz, 1983).

Preparation of Murashige and Skoog (MS) stock solutions

The formulation of Murashige and Skoog's (1962) medium is given in Table 4.1. Additional formulations are given at the end of the book. The MS basal salt mixture is commercially available in powder form from the list of suppliers in the end of the book.

Micronutrient stock (100×; Table 4.1C). Add approximately 400 cm^3 DDH_2O to a 1-liter beaker. Weigh and dissolve each of the salts given in the first column. Transfer the solution to a 1-liter volumetric flask, and add DDH_2O to the final volume. Store under refrigeration. Pipette 10 cm^3 of the micronutrient stock for 1 liter of MS nutrient medium.

Iron stock (20×; Table 4.1B). Add approximately 80 cm^3 DDH_2O to a 100-cm^3 beaker. Weigh and dissolve the two salts in the order indicated. Transfer the solution to a 100-cm^3 volumetric flask, and add DDH_2O to the final volume. Store at room temperature. Pipette 5 cm^3 of iron-stock for 1 liter of MS nutrient medium.

Vitamin stock (100×; Table 4.1D). Add approximately 50 cm^3 DDH_2O to a 100-cm^3 beaker. Weigh and dissolve each of the vitamins indicated. Transfer the vitamin mixture to a 100-cm^3 volumetric flask, and add DDH_2O to the final volume. Store under refrigeration. Because of the possibility of heat degradation of the vitamins, the vitamin supplement should be added after the remainder of the MS medium has been sterilized by autoclave. Add 1 cm^3 of sterile vitamin stock to each 100 cm^3 of autoclaved MS medium before the agar has cooled to the gelling point. At this time, the desired amount of vitamin stock can be sterilized by ultrafiltration with a syringe equipped with a sterile Swinney filter unit containing a Millipore filter disc (0.22-μm pore diameter).

Cytokinin stock (100×; Table 4.1E). Weigh 10 mg kinetin (K), and dissolve it in a few drops of 1 N HCl. Add a few cubic centimeters of DDH_2O, and transfer the solution to a 100-cm^3 volumetric flask. Add DDH_2O to the final volume. Store under refrigeration. Pipette 1 cm^3

Table 4.1. *Medium for Nicotiana tabacum stem callus*

Ingredient		Concentration[a]	
		Stock	MS medium
(A)	Macronutrients		mg/l
	$(NH_4)NO_3$		1,650
	KNO_3		1,900
	$CaCl_2 \cdot 2H_2O$		440
	$MgSO_4 \cdot 6H_2O$		370
	KH_2PO_4		170
(B)	Iron	mg/100 cm^3 (20×)	mg/l (5 cm^3 stock gives)
	Na_2EDTA	672	33.6
	$FeSO_4 \cdot 7H_2O$	556	27.8
(C)	Micronutrients	mg/l (100×)	mg/l (10 cm^3 stock gives)
	$MnSO_4 \cdot 4H_2O$	2,230	22.3
	$ZnSO_4 \cdot 4H_2O$	860	8.6
	H_3BO_3	620	6.2
	KI	83	0.83
	$Na_2MoO_4 \cdot 2H_2O$	25	0.25
	$CuSO_4 \cdot 5H_2O$	2.5	0.025
	$CoCl_2 \cdot 6H_2O$	2.5	0.025
(D)	Vitamins	mg/100 cm^3 (100×)	mg/100 cm^3 (1 cm^3 stock gives)
	glycine	20	0.2
	nicotinic acid	5	0.05
	pyridoxine·HCl	5	0.05
	thiamine·HCl	1	0.01
(E)	Cytokinin	mg/100 cm^3 (100×)	mg/l (1 cm^3 stock gives)
	kinetin	10	0.1
			mg/l
	myo-inositol		100
	IAA		10
	sucrose		30,000
	agar (0.8% w/v)		
	pH 5.7		

[a] In the 1962 publication ranges of concentrations were employed for IAA (1–30 mg/l) and kinetin (0.04–10 mg/l). In addition, a casein hydrolysate preparation (Edamin) was given as optional (1 mg/l). The concentration of Na_2EDTA was recalculated according to Singh and Krikorian (1980).

Source: Murashige and Skoog (1962).

of the cytokinin stock for 1 liter of MS medium to give a final concentration of 0.1 mg/l.

Note: Label all stock solutions with a high-melting-point wax pencil; include your initials and the date of preparation. Although inorganic salts are relatively stable in solution, the vitamin stock should be discarded after 30 days. Do not pipette directly from stock bottles, and do not return any unused stock solutions to the stock bottles.

Steps in the preparation of the MS medium (1-liter volume)

1. Add approximately 400 cm^3 DDH_2O to a 1-liter beaker. Weigh and dissolve each of the macronutrient salts given in Table 4.1A.
2. From each of the stock solutions previously prepared, add by pipette to the macronutrient solution: 5 cm^3 iron, 10 cm^3 micronutrient, and 1 cm^3 kinetin.
3. Weigh 100 mg *myo*-inositol, and dissolve it in the medium mixture.
4. Weigh 10 mg IAA, dissolve the auxin in a few drops of 1 N NaOH, and transfer it to the medium mixture. It is inadvisable to prepare an IAA stock solution because of the possibility of oxidative degradation of the hormone during prolonged storage.
5. Add DDH_2O until the total volume of liquid is approximately 800 cm^3. While stirring the solution, adjust the pH of the medium to 5.7 by delivering droplets of 1 N NaOH or 1 N HCl with separate Pasteur pipettes.
6. Transfer the medium to a 1-liter volumetric flask, and add DDH_2O to the final volume. Store under refrigeration. Label "MS, 10 IAA, 0.1 K, less vit."; sign your initials and the date of preparation. Use a high-melting-point wax pencil.

Final procedure

7. Sterilize the Petri dishes or culture tubes in advance with dry heat (see chap. 3). Several different sizes of culture tubes are available. Glass shell vials measuring 21 × 70 mm (15-cm^3 capacity) are excellent. Each vial can be poured with about 10 cm^3 medium, and 10 vials fit conveniently in a glass storage jar measuring 80 × 100 mm. The Petri dishes are wrapped with heavy-duty aluminum foil in packages of four or five. If tubes are used, each tube is capped with aluminum foil. The tubes must have a flat bottom so they will stand unsupported. The tubes are then placed in a glass container, and the entire unit is wrapped with aluminum foil.
8. Weigh 0.8 g Difco Bacto-Agar and 3.0 g reagent-grade sucrose, and transfer them to a 250-cm^3 Erlenmeyer flask. Add 100 cm^3 of the MS medium (step 6). Seal the flask with an aluminum foil cap, and sterilize the medium with wet heat (see chap. 3).
9. While the medium is in the autoclave, clean the interior of the transfer cham-

ber with a tissue soaked in 70% (v/v) ethanol. Arrange the sterile Petri dishes or culture tubes to receive the autoclaved medium.

10. After the sterilized medium is removed from the autoclave, permit the hot medium to cool in the chamber for 10–15 min before adding 1 cm^3 of the sterile vitamin stock. The vitamin preparation, sterilized by an ultrafiltration unit attached to the end of the syringe, is added to each flask. The flasks are swirled for a few minutes to ensure the dissolution of the sucrose and to mix the vitamins and agar with the remainder to the medium prior to pouring into the culture tubes. The Erlenmeyer flasks that contained the agar medium should be washed immediately after use (i.e., before the residual agar has solidified).
11. After the agar in the tubes has gelled, replace them in the storage jar and wrap the jar in aluminum foil. Store the tubes in the refrigerator until 1 hr before culture time.

Autoclaving of carbohydrates in a nutrient medium

Sucrose is prone to heat degradation during autoclaving. Sucrose-containing media sterilized in this manner will contain a mixture of D-glucose, D-fructose, and sucrose. The student should be aware of this because the MS medium previously described contains autoclaved sucrose. The growth and development of some plant tissues may be adversely affected by the presence of these monosaccharides. There is an alternative technique that will eliminate this problem. Obviously, the agar must be dissolved in liquid and autoclaved in order to bring it to the sol state; in addition, the interior of the flask must be sterilized.

Weigh 0.8 g Difco Bacto Agar, and transfer it to a 250-cm^3 Erlenmeyer flask. Add 50 cm^3 DDH$_2$O, cap the flask with foil, and sterilize by autoclave. During the cooling period, add to the flask 50 cm^3 MS medium (double strength) containing 3.0 g sucrose dissolved in the medium. The MS–sucrose mixture is sterilized by ultrafiltration prior to its addition to the agar in the flask.

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5

Initiation and maintenance of callus

A callus consists of an amorphous mass of loosely arranged thin-walled parenchyma cells arising from the proliferating cells of the parent tissue. Frequently, as a result of wounding, a callus is formed at the cut end of a stem or root. The term callus may be confused with "callose," another botanical term. The latter refers to a polysaccharide associated with sieve elements, and formed rapidly following injury to sieve elements and parenchyma cells (Esau, 1977). Although the major emphasis has been on angiosperm tissues, callus formation has been observed in gymnosperms, ferns, mosses, and liverworts (Yeoman, 1970; Yeoman and Macleod, 1977). Callus has no predictable organizational pattern, although localized centers of meristematic activity are present, and often a rudimentary cambial region appears with zones of vascular differentiation.

Sinnott (1960) has described some of the early observations on wound callus formation. The stimuli involved in the initiation of wound callus are the endogenous hormones auxin and cytokinin. In addition to mechanical injury, callus may be produced in plant tissues following an invasion by certain microorganisms (Braun, 1954) or by insect feeding (Pelet et al., 1960). Using tissue culture techniques, callus formation can be induced in numerous plant tissues and organs that do not ususally develop callus in response to an injury (Street, 1969). Plant material typically cultured includes vascular cambia, storage parenchyma, pericycle of roots, cotyledons, leaf mesophyll, and provascular tissue. In fact, all multicellular plants are potential sources of explants for callus initiation (Yeoman and Macleod, 1977).

In 1939 the first successful prolonged cultures of experimentally induced callus were achieved almost simultaneously at the research laboratories of Gautheret in Paris, Nobécourt in Grenoble, and White in Princeton. These cultures were originally derived from explants of cambial tissue of carrot and tobacco. The term "tissue culture," as applied to such cultures, is a misnomer. A cultured tissue does not maintain its

unique characteristics as a plant tissue, but reverts to an unorganized callus. The most important characteristic of callus, from a functional viewpoint, is that this abnormal growth has the potential to develop normal roots, shoots, and embryoids that can form plants.

The general growth characteristics of a callus involve a complex relationship between the plant material used to initiate the callus, the composition of the medium, and the environmental conditions during the incubation period. Establishment of a callus from the explant can be divided roughly into three developmental stages: induction, cell division, and differentiation. During the initial induction phase metabolism is stimulated as the cells prepare for division. The length of this phase depends mainly on the physiological status of the explant cells as well as the cultural conditions. Subsequently, there is a phase of active cell division as the explant cells revert to a meristematic or "dedifferentiated" state. A third phase involves the appearance of cellular differentiation and the expression of certain metabolic pathways that lead to the formation of secondary products. A detailed description of the growth patterns of callus cultures is given by Aitchison and his colleagues (1977). Certain aspects of the accumulation of secondary products as a facet of differentiation in callus cultures have been reviewed (Yeoman et al., 1982) and this topic will be discussed in Chapter 16. Some callus growths are heavily lignified and hard in texture, whereas others break easily into small fragments. Fragile growths that readily separate are termed "friable" cultures. Callus may appear yellowish, white, green, or pigmented with anthocyanin. Pigmentation may be uniform throughout the callus, or some regions may remain unpigmented. Anthocyanin-synthesizing and nonsynthesizing cell lines have been isolated from carrot cultures (Alfermann and Reinhard, 1971), and a stable pigment-producing strain of cultured *Euphorbia* sp. cells was isolated after 24 clonal selections and subcultures (Yamamoto, Mizuguchi, and Yamada, 1982). On the other hand, production of anthocyanin pigments by grape cell cultures was found to be unstable. Cultures started from white cells always contained some red cells, and red cell cultures invariably produced a mixture of both red and white cells (Yamakawa et al., 1982).

In regard to anatomy, there is considerable variability in the extent and type of cellular differentiation. A homogeneous callus consisting entirely of parenchyma cells is rarely found, although exceptions have been reported for cultures of *Agave* and *Rosa* cells (Narayanaswamy, 1977). Cytodifferentiation occurs in the form of tracheary elements, sieve elements, suberized cells, secretory cells, and trichomes. Small

nests of dividing cells form "meristemoids" or vascular nodules that may become centers for the formation of shoot apices, root primordia, or incipient embryos. Vascular nodules typically consist of discrete zones of xylem and phloem separated by a cambium. The orientation of the xylem and phloem with respect to the cambial zone is influenced by the nature of the original tissue (Gautheret, 1959; 1966). The location of the nodules within the callus can be modified by altering the composition of the medium (see chap. 7). Vascular differentiation may also take the form of a somewhat randomly arranged series of bundles or strands of tracheary elements (Roberts, 1976).

Early workers erroneously assumed that callus cultures derived from chlorophyll-containing organs would be autotropic in nutrition (Street, 1969). Hildebrandt and colleagues (1963) found that chlorophyllous callus was dependent on an exogenous sugar for continued growth, even with adequate light intensities. Reports by Bergmann (1967) and Edelman and Hanson (1971) provided evidence of photosynthetic activity of tissue cultures. By 1982 at least 12 photoautotropic cell cultures had been established from plants of a wide taxonomic distribution (Barz and Hüsemann, 1982). The first step in the establishment of photoautotropic cell cultures involves the selection of cell lines high in chlorophyll content and with a high degree of oxygen evolution (Yamada, Sato, and Hagimori, 1978). Several exogenous factors have proved beneficial for the culture of these selected cell lines: high light intensity, blue wavelengths of light for chloroplast differentiation and enzyme induction, low sugar content, low auxin levels, high CO_2, and increased levels of phosphate (see Barz and Hüsemann, 1982). Freshly isolated carrot root explants, cultured in the light on a medium supplemented with IAA, inositol, and kinetin, pass from heterotropic (up to 10 days of culture), through mixotropic (up to 21–3 days of culture), and eventually become autotropic in nutrition (Neumann et al., 11978). In experiments with cultures of tobacco and scotch broom (*Cytisus scoparius* Link) the labeled products of $^{14}CO_2$ fixation revealed that the green cells mainly fix CO_2 through the Calvin cycle, although C_4 organic acids are also formed in appreciable quantities (Yamada, Sato, and Watanabe, 1982). Similar results were reported for photoautotropic carrot cultures (Neumann et al., 1982).

One serious problem associated with the use of callus cultures, as well as other cell culture systems, is genetic instability resulting in variations in phenotypes within the cell population. Phenotypic variations arising during culture may have either a developmental (epigenetic) or a genetic basis. Epigenetic changes involve selective gene expression. These

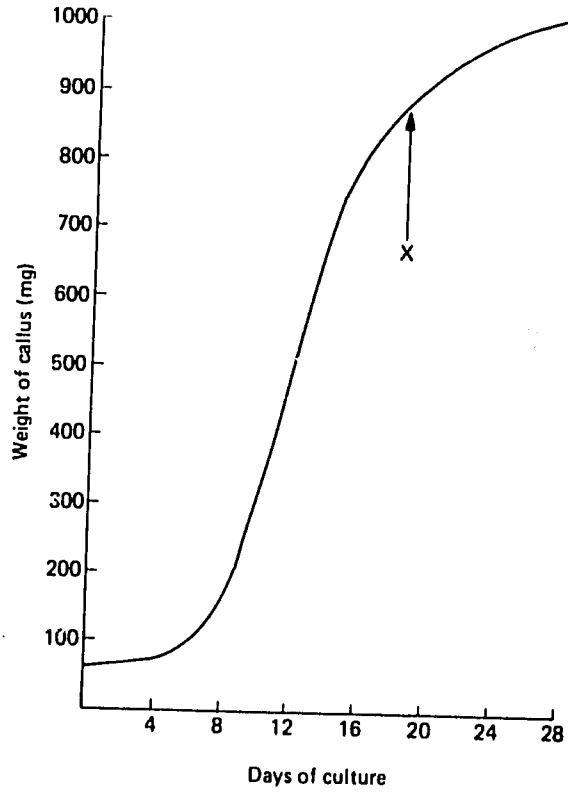
changes are stable, heritable at the cellular level, and are potentially reversible (Binns, 1981). The phenomenon of cytokinin habituation is an example of an epigenetic change that is well documented (Meins, 1983). Genetic variations involve changes in nuclear cytology (Sunderland, 1977). There may be chromosomal aberrations, nuclear fragmentation, and endoreduplication resulting in polyploidy (D'Amato, 1978). Often the cells of the initial explant prior to culture have genetic variability, and polysomatism is relatively common among the flowering plants (Constantin, 1981; Kasha, Kott, and Seguin-Swartz, 1982). The frequency of these nuclear abnormalities usually increases with the age of the culture, and the cultural conditions may act in a selective manner. Certain aneuploid or polyploid cells might gain an advantage in division rate over the normal cells, proliferate to a greater extent, and ultimately become the dominant cell line of the culture (Thorpe, 1982; Kasha et al., 1982). Variations that arise are also related to the composition of the culture medium, especially to the presence of hormones. Callus cultures of carrot and tobacco exhibit a significant level of polyploidy within a few months after explant isolation. Ploidy changes, however, may occur during callus initiation on the primary explant. Some callus subcultures of *Crepis capillaris* and *Helianthus annuus*, on the other hand, remain stable for periods of 2 years (Butcher and Ingram, 1976). Variability in plants regenerated in vitro was the subject of an international seminar in 1980 (Earle and Demarly, 1982).

The nutritional requirements for the initiation of callus vary considerably for primary explants of different origin. Juice vesicles from lemon fruits (Kordan, 1959) and explants containing cambial cells exhibit callus growth without the addition of any exogenous growth regulators. The majority of excised tissues, however, require the addition of one or more growth factors to the medium in order to stimulate callus development (Yeoman and Macleod, 1977). Explants can be subdivided, according to their growth factor requirements, in the following manner: (1) auxin, (2) cytokinin, (3) auxin and cytokinin, and (4) complex natural extracts. Supplements for callus initiation are given in Chapter 4.

After the callus has been grown for a period of time in association with the original tissue, it becomes necessary to subculture the callus to a fresh medium. Growth on the same medium for an extended period will lead to a depletion of essential nutrients and to a gradual desiccation of the agar because of water loss. Metabolites secreted by the growing callus may also accumulate to toxic levels in the medium. The transferred fragment of callus must be of a sufficient size to assure renewed growth on the fresh medium. If the transferred inoculum is too small, it may

exhibit a very slow rate of growth or none at all. Street (1969) has recommended that the inoculum be 5–10 mm in diameter and weight 20–100 mg. Successive subcultures are usually performed every 28 days with culture tubes containing 30 cm^3 medium. Passage time, however, is somewhat variable and depends on the rate of growth of the callus. A typical growth curve for callus cultures is shown in Fig. 5.1; it resembles growth curves plotted for bacterial cell cultures. Yeoman and Macleod (1977) suggest that cultures maintained on agar at 25°C or above should be subcultured every 4 to 6 weeks. A friable callus can be subdivided with a thin spatula or scalpel and transferred directly to the surface of the fresh medium. Hard callus growths must be transferred to the surface of a sterile Petri dish and sliced into fragments with a scalpel. Only healthy tissue should be transferred, and brown or necrotic tissue must be discarded. Interest has been shown in developing alternative methods

Fig. 5.1. Growth response of a typical callus culture. This particular callus should be subcultured approximately at the time indicated by X.



for long-term maintenance of tissue cultures, for example, freeze preservation (Withers, 1979; see chap. 15).

The purpose of the following experiment is to acquaint the student with the basic technique of inducing callus in a primary explant excised from the taproot of carrot (*Daucus carota* L.). The newly formed callus will be subcultured and attempts made to maintain the growth of the callus during three successive subcultures. Fresh carrots are readily available in markets throughout the year, and this plant material has served as the basis for a prodigious number of research investigations. Gautheret (1959) has described the characteristics of callus formation by carrot root tissues. Fragments of primary phloem proliferate weakly and produce either isolated pustules of cells or a thin layer of parenchyma cells. Explants of xylem exhibit two types of responses, depending on the origin of the explant. If the explant is removed from the vicinity of the vascular cambium, the most recent derivatives of the cambium divide vigorously to form extensive callus. Removal of xylem from the central region of the root results only in isolated callus at the extremities of the vessels. Explants of secondary phloem bordering the vascular cambium produce the most vigorous growth. The student should become familiar with the anatomy of the carrot taproot before starting the experiment.

LIST OF MATERIALS

Sterilization mode: C, chemical; O, oven; A, autoclave

C large and healthy taproot of carrot (*Daucus carota* L.) fresh from the market (see appendix for alternative plant material)
paring knife; vegetable scraper
300 cm^3 aqueous solution (10% v/v) commercial bleach containing a final concentration of approximately 0.5% (v/v) NaOCl

O 600-cm^3 beakers (four)

O 9-cm Petri dishes (five)

O stainless steel cork borer (no. 2) containing a metal rod.
Enclose borer in a test tube capped with aluminum foil, and place an additional layer of foil around the entire unit.

O stainless steel forceps. Enclose forceps in a test tube and wrap with foil in a manner similar to that of the cork borer.

O culture tubes, 21 × 70 mm (15 cm^3 capacity) recommended (30). Cap tubes with aluminum foil, place them in glass storage jars (80 × 100 mm), and wrap the unit with aluminum foil.

A 9-cm Petri dishes, each containing two sheets Whatman No. 1 filter

paper (five). Enclose the dishes in a paper bag and mark the letter F on the paper with a wax marking pencil.

A 125-cm^3 Erlenmeyer flasks, each containing 100 cm^3 DDH_2O (12).

A 300 cm^3 of MS callus-induction medium supplemented with sucrose (3% w/v) and Difco Bacto-Agar (0.8% w/v). Prepare 3 × 100-cm^3 aliquots in 250-cm^3 Erlenmeyer flasks.

A 9-cm Petri dish equipped as explant cutting guide (see appendix for construction). Enclose the dish in a paper bag and mark the letter C on the paper with a wax marking pencil.

C scalpel. Bard-Parker no. 7 surgical knife handle equipped with a no. 10 blade is recommended.

ethanol (80% v/v) dip for flaming instruments. Place the dip in a metal can as a fire precaution.

ethanol (70% v/v) in plastic squeeze bottle for surface sterilization of transfer chamber

methanol lamp

interval timer

heavy-duty aluminum foil (one roll)

light microscope (100 × magnification)

dissecting microscope or hand lens

dissecting needles

microscope slides; cover slips

lens paper

aqueous solution toluidine blue O (0.05% w/v) in dropper bottle

Pasteur pipette

PROCEDURE

Follow the outline given in the appendix of Chapter 4 for the preparation of 1 liter of MS medium supplemented with IAA (10 mg/l), kinetin (0.1 mg/l), sucrose (3% w/v), and agar (0.8% w/v). Prepare to culture 30 carrot explants, which will require the preparation of 30 culture tubes containing the medium (10 cm^3 each).

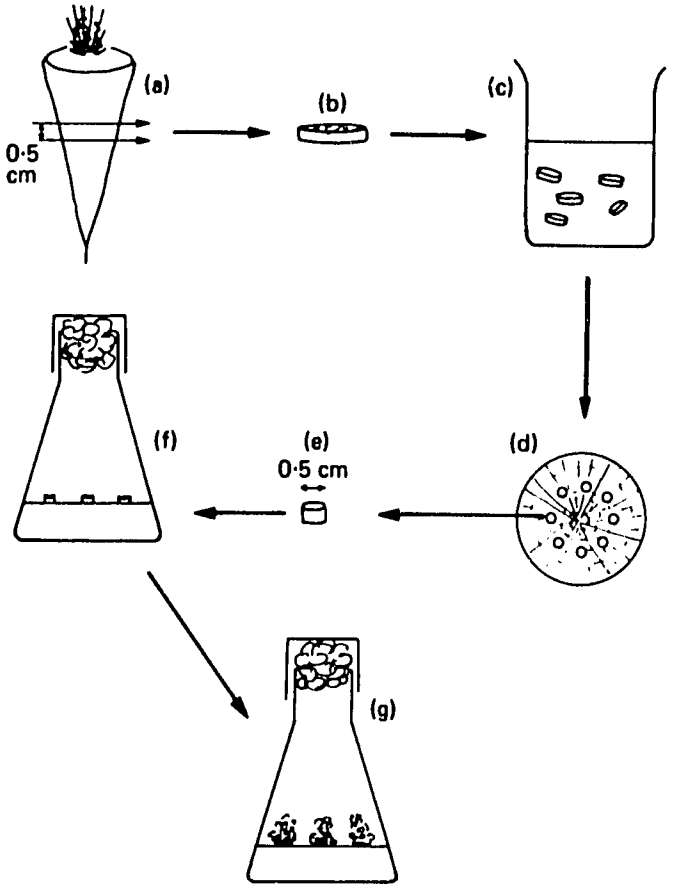
If the experiment is conducted as a classroom project, each student may be assigned a different combination of auxin and cytokinin. Another possible assignment is to examine the relative effectiveness of different concentrations of IAA and kinetin on the induction of callus growth. In any experiments involving comparisons of callus growth, the explants must be removed from the same tissue zone in the taproot.

1. The isolation of cambial explants from the taproot is shown diagrammatically in Fig. 5.2. Wash the taproot thoroughly under running

tap water, and remove the external 1–2 mm of tissue with a vegetable scraper. Cut the carrot transversely into slices approximately 10 mm in thickness, and immediately place the slices in a beaker of tap water. All subsequent steps must be carried out under aseptic conditions.

2. Surface sterilize the working area within the transfer chamber with

Fig. 5.2. Diagram showing the preparation of explants from the cambial region of carrot (*Daucus carota*) taproot. (a,b) A segment approximately 0.5 cm in thickness is removed from the taproot. (c) The segments are surface sterilized and subsequently rinsed repeatedly in sterile DDH_2O (not shown). (d,e) Short cylinders of tissue (0.5 cm O.D.) are removed with a sterile cork borer from the cambial zone of the taproot. (f) After trimming the ends of tissue that may have been injured by the sterilization, the explants are placed on the surface of the agar-solidified medium. (g) Following incubation at a suitable temperature, callus will arise from repeated divisions of the cultured cells.



a tissue soaked with ethanol (70% v/v). Place the carrot slices in one of the sterile 600-cm^3 beakers. Add the hypochlorite solution to the beaker, and set the timer for 10 min. If the chamber is equipped with an ultraviolet lamp *do not* turn on the lamp. Ultraviolet-induced degradation of the hypochlorite solution might occur (see chap. 3).

3. After approximately 8 min, preparations can be started for rinsing the slices. Thoroughly wash you hands with soap and hot water before starting the aseptic procedure. Unwrap the remaining three beakers (600 cm^3), and add approximately 300 cm^3 DDH_2O to each beaker. Following the 10-min sterilization period, remove the slices from the hypochlorite solution with forceps, and rinse then successively 20–30 sec in each of the three rinse beakers. Withdraw from the chamber the beakers containing the hypochlorite solution and the first two rinses. Light the methanol lamp, and place the scalpel in the ethanol dip. Open the foil packet containing the cork borer and the packet of empty Petri dishes. Place two of the dishes in the rear of the chamber, and fill each dish about half-way with sterile DDH_2O. Partially remove the lid of one of the dishes. Remove the lid of the third empty Petri dish, exposing the sterile surface of the lower half.

4. Flame the forceps and transfer one of the carrot slices from the final rinse beaker to the surface of the exposed Petri dish (boring platform). Steady the slice with the forceps, and make a boring with the cork borer parallel to the vertical axis of the tissue slice. The boring should be made from the region of the vascular cambium (Fig. 5.2d). The cork borer must be inserted all the way so that it cuts completely through the tissue. Lift the tissue slice with the borer still inserted in it, and hold the slice directly over the DDH_2O in the partially opened Petri dish. Gently exert pressure on the metal rod. This slight force should eject the tissue cylinder into the pool of water. Return the slice to the boring platform. Place the arms of the forceps on each side of the borer, and withdraw the borer from the slice. Repeat the process until you have prepared a maximum number of tissue cylinders from the cambial zone of the slice. Discard the tissue slice in the lid of the boring platform, and prepare additional cylinders from the remaining carrot slices. Finally, withdraw from the chamber the final rinse beaker, cork borer, remains of the carrot slices, and the boring platform.

5. Open the packet marked C containing the explant cutting guide (see Appendix). If this device is unavailable, the base of a sterile Petri dish can be used for slicing explants from the cylindrical tissue borings. Arrange the following three Petri dishes in the rear of the transfer chamber. The nearest dish should be the explant cutting guide. Another dish

will contain the cylinders of tissue, and the third sterile DDH_2O for explant rinsing (prepared in step 3). Partially open the two dishes in the rear of the chamber, and completely remove the lid of the explant cutting guide.

6. Flame the forceps and scalpel, and transfer a tissue cylinder with the forceps to the cutting guide. It is important to permit flamed instruments to cool briefly before bringing them into contact with living plant tissues. Trim and discard approximately 2 mm of tissue from each end of the cylinder. Slice the remaining cylinder into three pieces approximately 2 mm in length. Each explant will measure about 5 mm in diameter and 2 mm in thickness. With the flat blade of the scalpel, transfer the explants to the dish containing DDH_2O rinse. Repeat the cutting operation until all of the cylinders have been sliced into explants. Flame the forceps and scalpel several times during the course of the slicing operation. Remove from the chamber both the Petri dish that contained the cylinders of tissue and the cutting guide.

7. Fill an empty Petri dish halfway with DDH_2O. Flame the forceps and transfer the explants to the dish containing the rinse water. Remove from the chamber the dish that formerly contained the explants. Open the paper bag marked F containing Whatman No. 1 filter paper. Arrange the culture tubes to receive the explants.

8. Flame the forceps, and transfer the explants one at a time to the surface of the sterile filter paper. Blot briefly both the top and bottom surfaces of each explant. Immediately transfer the explant to the surface of the culture medium (one explant per tube). Remember to hold the culture tubes at a slight angle so that the hand grasping the forceps is not directly over the sterile surface of the medium.

9. The culture tubes containing the explants are placed in glass storage jars, and the jars are wrapped in aluminum foil. Transfer the cultured explants to an incubator adjusted to 25°C. If a controlled-temperature facility equipped with fluorescent lighting is available, the comparative effect of light versus dark on callus initiation can be examined. According to Street (1977), "Too little is, however, known about the effect of low light intensities on culture growth to suggest any optimum intensity level or spectral composition for general culture maintenance."

RESULTS

After a few days in culture the explants become slightly rough in texture, and the surface of the explants may glisten in reflected light. This is a sign of the beginning of callus formation. Culture for a single incubation

period (passage) may last from a few weeks to 3 months, depending on the rapidity of growth (Thomas and Davey, 1975). The callus is then subdivided into fragments that serve as inocula for the fresh medium. Depending on the friability of the callus, use either a spatula or a scalpel for removing inocula from the parent callus mass. Only healthy tissue can be used; brownish tissue is a sign that localized necrosis has occurred. The instruments must be flamed and aseptic techniques used throughout the subculture procedure.

Examine the surface of the callus with a dissecting microscope or a hand lens, and notice the external appearance of the newly formed cells. With a dissecting needle scrape some of the cells onto a microscope slide. Add a drop of distilled water and a cover slip, and examine the cells with the light microscope (100 × magnification). The contrast can be enhanced by lightly staining the callus cells with an aqueous solution of toluidine blue O (0.05% w/v; McCully and O'Brien, 1969). With a Pasteur pipette add a microdrop of the stain solution to one edge of the cover slip. On the opposite side of the cover slip moisten a piece of lens paper with the aqueous mounting medium. The blotting action of the paper will draw the stain beneath the cover slip and into the field of vision.

QUESTIONS FOR DISCUSSION

1. Why are explants containing cambial cells excellent plant material for the initiation of callus?
2. Some callus growths gradually lose the need for exogenous auxin and/or cytokinin for sustained growth. What term is used to describe this phenomenon? What is the significance of this change in nutritional requirements?
3. Under the cultural conditions employed, what was the optimal interval of time between subcultures?
4. Why do callus cultures become brown and necrotic if they are left too long on the same medium?
5. What evidence shows that cells of carrot callus retain the genetic "master plans" of the original carrot plant?

APPENDIX

Alternative plant material. Nearly all plant tissues and organs containing living cells may be induced to initiate callus, although different tissues vary in the lag periods before active growth occurs (Gresshoff, 1978). Callus is easily started from explants of soybeans (*Glycine max*) cotyledons after surface sterilization of the seeds. Another approach is to germinate the seeds under aseptic conditions after surface sterilization. Ex-

plants are prepared from the hypocotyl or other part of the axenic seedlings. Several market vegetables can be used for the induction of callus: potato tuber (*Solanum tuberosum*), storage root of sweet potato (*Ipomea batatas*), hypocotyl of radish (*Raphanus sativus*), and storage root of turnip (*Brassica rapa*). Explants of potato tuber, in particular, produce excellent callus (Anstis and Northcote, 1973; Shaw et al., 1976). If greenhouse space is available, tobacco plants should be grown to maturity. The stem pith of tobacco (*Nicotiana tabacum*) is one of the best tissues for callus formation.

Explant cutting guide. An aid for slicing explants can be constructed from a Petri dish, a glass rod, and an index card. Cut a small-diameter glass rod to a slightly smaller length than the inside diameter of the dish, and cement the rod to the bottom half of the dish. On a 3- × 5-inch index card, draw two parallel lines approximately 2.5–3.0 mm apart. Trim the card and tape it to the outside of the bottom half of the dish with one of the lines parallel to the glass rod and directly beneath it. During explant preparation the cylinder of tissue is pressed against the rod and held perpendicular to it. The line on the card acts as a visual guide to indicate the position of the scalpel in cutting explants of approximately the same thickness. Wrap the device in a paper bag and mark the letter C on the paper with a wax pencil. This will distinguish it from the Petri dishes containing filter paper in the other bag (marked F). Sterilize the cutting guide in the autoclave, and place it briefly in the drying oven (low temperature) to evaporate the condensed moisture from the autoclave.

Cork borer. The cutting edge of the cork borer can be maintained by occasionally rubbing it with a fine grade of abrasive paper. In the United States the Wetordry Tri-M-ite papers (400 and 600 grades) have given satisfactory results.

Cytokinin properties of coconut milk. Repeat the experiment as outlined in this chapter, except employ three different media. Each medium is prepared so as to contain a different combination of plant growth regulators: (1) IAA (10 mg/l) and kinetin (0.1 mg/l), (2) IAA (10 mg/l) alone, and (3) IAA (10 mg/l) plus coconut milk (10% v/v). The procedure for the preparation of coconut milk from fresh coconuts is given in the appendix of Chapter 4. Can you detect any differences in the appearance of callus arising from the explants on the three different media? In your opinion does this experiment provide evidence for the presence of a cytokininlike substance in coconut milk?

Auxin habituation. During the second or third subculture, add some callus inocula to culture tubes containing a nutrient medium lacking exogenous auxin. Does the carrot callus on the auxin-deficient medium exhibit growth? Experimental evidence suggests the carrot cultures may beome auxin habituated following an initial "pulse" of exogenous auxin (Bender and Neumann, 1978).

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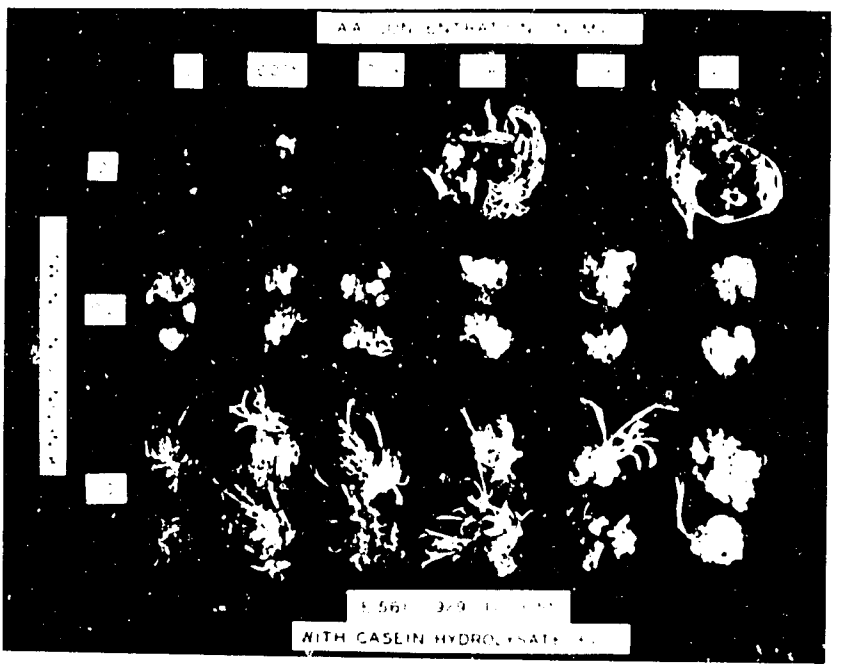
6

Organogenesis

Although observations were made on the formation of organs in vitro since the early studies on plant tissue cultures, there is still a paucity of factual information on the induction of organogenesis in isolated and cultured plant tissues. Aside from the basic research efforts of Thorpe and his colleagues, most investigations continue to be centered about an empirical manipulation of the three controlling factors, the inoculum, the medium, and the environmental conditions (Thorpe, 1980; 1982a). Roots, shoots, and flowers are the organs that may be initiated from plant tissue cultures. Embryos are not classified as organs because these structures have an independent existence (i.e., embryos do not have vascular connections with the parent plant body). In a given callus only a few cells are involved in the initiation process, and the onset of the process is asynchronous and somewhat unpredictable. The underlying factors involved in organogenesis are obscure because the stimuli may involve components of the medium, endogenous compounds produced by the culture, and substances carried over from the original explant (Thomas and Davey, 1975). With prolonged subculture of callus several changes may occur in the system. These include hormone habituation, the loss of organogenic potential, and changes in the external characteristics of the callus such as friability (Thorpe, 1980). The first indication that in vitro organogenesis could be chemically regulated, to some extent, was given by Skoog (1944). He found that the addition of auxin to the medium served to stimulate root formation, whereas shoot initiation was inhibited. The latter effect on shoot production could be partially reversed by increasing the concentration of both sucrose and inorganic phosphate (Skoog, 1944). Subsequently, it was found that adenine sulfate was active in promoting shoot initiation, and this chemical reversed the inhibitory effect of auxin (Skoog and Tsui, 1948). The studies of Skoog and his colleagues led to the hypothesis that organogenesis is controlled by a balance between cytokinin and auxin. A relatively high

auxin:cytokinin ratio induced root formation in tobacco callus, whereas a low ratio of the same hormones favored shoot production (Fig. 6.1; Skoog and Miller, 1957). Probably the most precise regulation of organ formation has been achieved with epidermal and subepidermal explants consisting of a few cell layers in thickness (Tran Thanh Van, 1980a,b). The formation of floral buds, vegetative buds, and roots has been demonstrated in thin cell-layer explants of several species by regulating the auxin:cytokinin ratio, carbohydrate supply, and environmental conditions (Tran Thanh Van, Chlyah, and Chlyah, 1974; Tran Thanh Van and Trinh, 1978). Certain isolated tissue layers, in species that readily regenerate organs in vivo, showed a remarkable potential to form organs during culture. Primary explants consisting of three to six cell layers of epidermal and subjacent collenchyma removed from the region of the leaf midvein of *Begonia rex* produced shoots or roots from the epidermal cells (Chlyah and Tran Thanh Van, 1975; Tran Thanh Van, 1980a). Root

Fig. 6.1. The regulation of organ formation in explants of tobacco (*Nicotiana tabacum*) pith by varying the auxin:cytokinin ratio. Note the occurrence of shoots induced by high levels of kinetin. Moderate levels of kinetin, in the presence of auxin, stimulate the production of callus. Auxin induced root formation in the absence of kinetin. (Courtesy of F. Skoog.)



initiation occurred in the presence of NAA plus zeatin, and shoot formation required the addition of either zeatin or benzylaminopurine in the absence of auxin. Organogenesis has been shown to occur in several other species from the epidermal layer during culture (Tran Thanh Van and Trinh, 1978).

Torrey (1966) advanced the hypothesis that organogenesis in callus starts with the formation of clusters of meristematic cells (meristemoids) capable of responding to factors within the system to produce a primordium. Depending on the nature of the internal factors, the stimuli can initiate either a root, a shoot, or an embryoid. Many observations on organ formation in cultured tissues support the hypothesis that localized meristematic activity precedes the organized development of roots and shoots (Ross, Thorpe, and Costerton, 1973; Maeda and Thorpe, 1979). The factors that regulate the origin of these meristematic zones are not understood. Since these zones are located in the vicinity of the tissue–medium interface, it has been suggested that physiological gradients of substances diffusing from the medium into the tissue may play a role in determining the loci at which meristemoids are formed (Ross et al., 1973). The meristematic region may also act as a sink and withdraw essential metabolites from the surrounding cells, thus localizing the meristematic zone (Street, 1977).

Root initiation is the type of organogenesis most frequently found in cultured tissues. A distinction should be made between the rooting of shoots under in vitro conditions, and rhizogenesis from meristemoids in callus cultures. Root initiation frequently occurs after the cultured tissue has produced buds, and shoot development undoubtedly alters the endogenous hormones within the culture (Gresshoff, 1978). Root formation at the base of cultured shoot apices is an important aspect of micropropagation, and this topic will be discussed in Chapter 10. The controlling factors involved in root formation in Jerusalem artichoke callus include mineral salts, sugar, auxin, temperature, and light (Gautheret, 1969). In some cultured tissues auxin promotes root formation, whereas in other systems, exogenous auxin is inhibitory, and rhizogenesis can be stimulated by antiauxin (Thomas and Street, 1970). Such reports are not contradictory when one realizes that it is the endogenous auxin:cytokinin balance that is the key factor in the initiation of the process (Thorpe, 1980). The initiation of roots in callus is often too sporadic for purposes of experimentation. The potential to form roots may decline after several subcultures, but the reason for this loss of morphogenetic potential is unknown. It may be due to the exhaustion

of a specific factor present in the initial explant and no longer synthesized in vitro (Gautheret, 1966). Another possible explanation for the loss of this characteristic is that epigenetic changes involving selective gene expression have occurred in the cultured tissues (Meins, 1983). The appropriate culture conditions for root formation in some species or variety may be completely ineffective in a closely related organism (Street, 1977). Several investigators have evidence of phenolic compounds acting in combination with auxin to promote organogenesis (Thorpe, 1980). The combination of phloroglucinol and indolebutyric acid, for example, was much more effective in stimulating rooting than auxin alone (Welander and Huntrieser, 1981). Apparently there is no direct relationship between ploidy changes and rhizogenesis, since the morphogenetic potential of *Brachycome* callus was lost without any change in the chromosomal content of the cultured cells (Gould, 1978).

Shoot initiation or caulogenesis in cultured plant tissues can be induced in many systems by an appropriate balance of exogenous auxin and cytokinin, and, in some cases, either one or the other of these growth regulators must be omitted from the medium in order to produce buds (Street, 1977). Buds are initiated in many dicot callus cultures by transferring the callus to a medium containing a cytokinin:auxin ratio in the range of 10 to 100, whereas callus production is favored by an auxin:cytokinin ratio of approximately 10 to 100. The term "reversal transfer" has been applied to the transfer of a culture from a callus-supporting medium to a shoot-inducing medium (Murashige and Nakano, 1965; Gresshoff, 1978). Unlike the conventional tobacco system, monocot callus derived from alfalfa (*Medicago sativa*) produces roots after a 4-day exposure to a high kinetin:2,4-D ratio. Shoots are formed by a similar treatment involving the same exposure to a relatively high 2,4-D:kinetin ratio. Organogenesis does not occur on the induction medium, but is initiated only after transfer of the callus to a medium devoid of growth regulator (Walker, Wendeln, and Jaworski, 1979). With other monocot cultures exogenous cytokinin may be unnecessary for the initiation of buds. The omission of auxin from the medium may be sufficient to induce shoot formation in these cultures, and two successive transfers on auxin-free media are recommended (Gresshoff, 1978). In addition to cytokinins, a variety of related compounds are capable of replacing cytokinins for shoot induction. These chemicals include substituted purines, pyrimidines, and ureas (Thorpe, 1980). In addition, adenine sulfate is capable of functioning as a cytokinin in the induction of buds (Skoog and Tsui, 1948; Sterling, 1951).

Auxin:cytokinin supplements or omissions from the medium hav failed to induce shoots in the culture of many species, and Street (1977 has offered some possible reasons for these failures: (1) Additional hor mones may be required; (2) endogenous hormones may accumulate, an their inhibitory effect on organogenesis is not reversed by the exogenou hormones; and (3) cultural conditions involving nutritional and physica factors may block the onset of the process.

The relative concentration and spectrum of endogenous gibberellin like compounds change during shoot initiation, and this group of hor mones plays a role in organogenesis (Thorpe, 1980). Gibberellins, i general, tend to suppress shoot and root formation. Shoot-forming callu accumulates starch, and this biosynthetic process is thought to be re quired for bud initiation. The inhibitory effect of GA_3 on caulogenesi has been related to the lowering of the starch content of the bud-formin cells (Thorpe, 1978; Maeda and Thorpe, 1979).

Endogenous ethylene may be a factor in caulogenesis. Early in culture this gaseous hormone blocks organogenesis, but during primordia for mation ethylene enhances their development (Huxter, Reid, and Thorpe 1979). Endogenous ethylene was identified as a factor in the inductior of buds from cultured tobacco cotyledons (Everett, 1982). Indirect ev idence suggests a similar role for ethylene in cultured *Lilium* bulb tissue (Aartrijk and Blom-Barnhoorn, 1983). Additional information on the organogenetic effects of growth-active substances can be found in the review by Thorpe (1980).

Carbohydrate metabolism is another factor to be considered in caulo genesis (Thorpe, 1982b). In addition to serving as a respiratory energy source, the exogenous carbohydrate may act as an osmotic agent. Mild osmotic stress apparently causes biochemical alterations that are reflected in changes in callus growth and morphology. The partial replacement of the sucrose requirement for bud formation in tobacco callus with mannitol supports the view that at least part of the carbohydrate in the system is performing an osmoregulatory function (Brown, Leung, and Thorpe, 1979). Murashige (1977) also stressed the importance of high osmolarity, as well as light and chelating agents, in shoot regeneration.

In some cultured tissues an error occurs in the developmental programming for organogenesis, and an anomalous structure is formed. Internodal stem segments of *Quercus rubra* L. seedlings produced "organoids" on callus arising from the primary explant and the subcultured callus (Seckinger, McCown, and Struckmeyer, 1979). Although these structures contained the dermal, vascular, and ground tissues present in

plant organs (Esau, 1977), they differed from true organs in that the organoids were formed directly from the periphery of the callus and not from organized meristemoids. The organoids proliferated in culture, and adventitious roots were formed from them (Seckinger et al., 1979). Similar types of anomalous structures have been observed on the callus of a different genus in the Fagaceae (Keys and Cech, 1978), on bacteria-free cultures of crown gall of *Nicotiana* (Braun, 1959), and on normal tissues of *Taraxacum* roots (Bowes, 1971).

In the present experiment the student will attempt to induce the formation of plantlets from leaf explants of *Saintpaulia ionantha* Wendl. (African violet). This plant has been propagated in vitro from explants of leaf lamina (Start and Cummings, 1976; Cooke, 1977; Vasquez, Davey, and Short, 1977), petioles (Bilkey, McCown, and Hildebrandt, 1978; Harney and Knap, 1979), and floral organs (Hughes, 1977; Vasquez et al., 1977). Harney (1982) has given a résumé of the in vitro propagation procedures for the regeneration of African violets.

LIST OF MATERIALS

Sterilization mode: C, chemical; O, oven; A, autoclave

C leaves excised from mature plant of African violet
200 cm^3 aqueous solution (10% v/v) commercial bleach containing a final concentration of approximately 0.5% (v/v) NaOCl. Add a few drops of a liquid detergent as a wetting agent.
O 250-cm^3 beakers (five)
O stainless steel forceps. Place forceps in test tube and wrap with foil prior to sterilization.
O 9-cm Petri dishes (five), foil wrapped
O culture tubes, 21 × 70 mm (15 cm^3 capacity) recommended (20)
A 9-cm Petri dishes, each containing two sheets Whatman No. 1 filter paper (five). Enclose the dishes in a paper bag and mark the letter F on the paper with a wax marking pencil.
A 125-cm^3 Erlenmeyer flasks, each containing 100 cm^3 DDH_2O (8)
A 200 cm^3 of MS medium, supplemented as given in the procedure. Prepare 2 × 100-cm^3 aliquots in 250-cm^3 Erlenmeyer flasks.
C scalpel. Bard-Parker no. 7 surgical knife handle equipped with a no. 10 blade is recommended.
ethanol (80% v/v) dip stored in a metal can as a fire precaution
ethanol (70% v/v) in plastic squeeze bottle
methanol lamp

interval timer
heavy-duty aluminum foil (one roll)
plant growth chamber (25°C)
pots; sterile soil mixture

PROCEDURE

Prepare 1 liter of MS medium as outlined in Chapter 4, and supplement with *myo*-inositol (100 mg/l), nicotinic acid (0.5 mg/l), pyridoxine-HCl (0.5 mg/l), thiamine-HCl (0.4 mg/l), NAA (0.1 mg/l), BAP (5.0 mg/l), and adenine sulfate (80 mg/l). Adjust the pH of the final medium to 5.7. The experiment as outlined requires 200 cm^3 of the medium, and this is prepared in two 100-cm^3 aliquots. Each aliquot will contain sucrose (3.0% w/v) and agar (0.7% w/v). The autoclaved medium is poured into 20 culture tubes (10 cm^3 each).

Culture procedure

1. Excise several healthy leaves, both young and old, and discard the petioles. Wash the blades or laminas briefly in cool soapy water. Rinse them in running tap water, and prepare for aseptic procedures. Because the steps in the preparation of the plant material for organogenesis follow the same basic procedure as outlined in Chapter 5, the following instructions will be given in abbreviated form.

2. Dip the blades in ethanol (70% v/v) and rinse in sterile DDH_2O in a 250-cm^3 beaker. Complete the surface sterilization by immersing the blades in the hypochlorite solution for 10 min. This two-step disinfestation is considered necessary because of the abundance of epidermal hairs on the surface of the blade. Rinse the blades in 3 successive baths of DDH_2O.

3. Each blade is transferred to a sterile Petri dish containing filter paper, and explants are prepared with forceps and scalpel. The filter paper will remove the excess moisture from the final DDH_2O rinse. The blade tissue most effective in organogenesis is located in the central part, and the outer margins and leaf tip are relatively unproductive. Slice the blade into rectangles approximately 10–12 mm on a side, ensuring that each explant contains a portion of the midvein of the leaf. Place the explants individually in culture tubes in an upright position with one-quarter of the explant embedded in the agar medium (Start and Cummings, 1976).

4. Place the cultures in a plant growth chamber maintained at 25°C

with 16-hr photoperiods furnished by a combination of Gro-Lux and cool-white fluorescent tubes. The light intensity should be approximately 1,000–1,500 lux.

RESULTS

Shoots will appear within 2 to 4 weeks, and the initial shoots are usually associated with the severed veins on the adaxial surface of the blade. After 6 to 8 weeks of culture the proliferated shoots can be aseptically subdivided and subcultured for the initiation of roots. Rooting is promoted by transferring the shoots to a medium that is devoid of plant hormones and has a sucrose concentration of approximately 1.6% (w/v) (Start and Cummings, 1976). Some research workers believe that this subculture step is unnecessary, since the subdivided shoots will readily establish a root system in a sterile potting soil mixture (Harney and Knap, 1979). A small bag of sterile African violet soil mixture can be purchased from a local supermarket or florist shop. The miniature pots should be maintained under a relatively high humidity with adequate lighting. Direct sunlight, however, can be harmful. The requirements for hardening off the plantlets are similar to those for plantlets regenerated by the micropropagation of the shoot apex (see chap. 10).

QUESTIONS FOR DISCUSSION

1. Can you think of any observations made on the production of shoots by the cultured leaf explants of African violet that suggest endogenous factors may play a role in the initiation of bud primordia?
2. An unsuccessful attempt was made to induce shoot formation in a dicot callus culture by supplementing the medium with a high cytokinin:auxin ratio. What are some alternative techniques that might induce shoot formation in this culture?
3. What is a meristemoid? Is a meristemoid considered totipotent?
4. In regard to organogenesis, what unusual characteristic was shown by an alfalfa callus culture?
5. What possible relationship exists between caulogenesis and the inhibition of this developmental process by gibberellins?

APPENDIX

Plantlets from tomato leaf explants (Padamanabhan, Paddock, and Sharp, 1974). Prepare leaf explants from young leaves removed from near the shoot apex of tomato (*Lycopersicon esculentum*) plants by cutting rectangles of surface-sterilized tissue 6 × 8 mm in size. Culture each explant in 15 cm^3 of liquid medium composed of MS mineral salts, thiamine (0.4 mg/

l), *myo*-inositol (100 mg/l), sucrose (30,000 mg/l), and a growth regulator supplement. The optimum concentrations of plant growth regulators for root initiation are IAA (2 mg/l) plus kinetin (2 mg/l). Incubate the cultures in a growth chamber set at 25°C on 12-hr photoperiods. Callus is initiated after approximately 8–10 days of culture. Following rhizogenesis, transfer the cultures to a similar medium containing IAA (4 mg/l) plus kinetin (4 mg/l). Shoot formation occurs after about 4 weeks, and plantlet formation is then facilitated by transferring the cultures to a basal MS medium without exogenous plant growth regulators. It may be necessary to prepare subcultures of the callus every 3–4 weeks during the course of the experiment.

Shoot initiation on stem explants of Populus sp.(R. R. Willing, unpublished). Remove a first-year twig from a poplar (*Populus* sp.). Prepare an internode explant 1–2 cm in length and split the stem segment lengthwise. Place the segments, arranged with the cut surfaces of the bark uppermost, in a Petri dish containing Whatman No. 1 filter paper wetted with an aqueous solution of kinetin (2.0 mg/l). After a few weeks callus will be evident, and shortly thereafter bud formation occurs. This is a simple experiment to perform because aseptic conditions are unnecessary. The student should attempt to induce rhizogenesis and the regeneration of poplar plantlets. The production of callus by isolated segments of poplar stem "cultured" on wet filter paper was first observed by Rechinger in 1893.

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7

Induction of the differentiation of xylem cells

Cytodifferentiation refers to a morphological and biochemical specialization of a given cell, as well as the developmental process leading to this unique condition. In the latter sense, differentiation has been defined as the summation of processes leading to the acquisition of specific metabolic competences (or the loss thereof) that serve to distinguish daughter cells from each other and from the parental cell (Gross, 1968).

The basic problem of differentiation centers around the genetic reprogramming of a cell in order to produce these metabolic changes. Determination refers to the process by which a cell becomes restricted to a new pathway of specialization; the transient period during which the new cytodifferentiation is initiated has been termed the point of determination (Roberts, 1976).

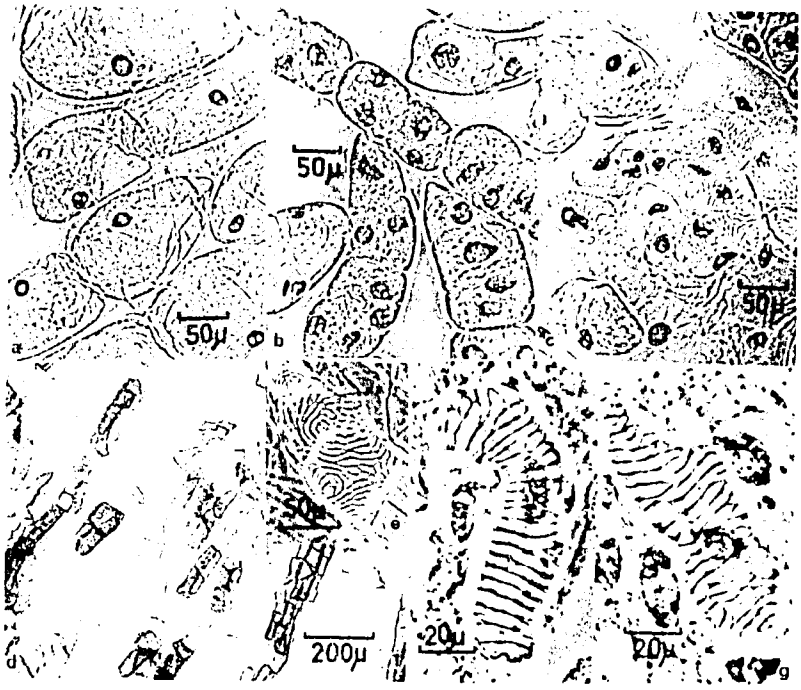
The xylem cell has been selected as a model system for studying cytodifferentiation in plants for several reasons. With the exception of sieve elements, few other cell types can be experimentally induced to form under the conditions of plant tissue culture. Xylem cells are easily identified with the light microscope because of the unusual pattern of secondary-wall thickening (Fig. 7.1). Cytodifferentiation occurs at a relatively rapid and predictable rate, and newly formed xylem cells first appear in Jerusalem artichoke tuber explants after 48 hr, and after 4 and 5 days in cultures of lettuce pith and pea root, respectively (Phillips, 1980). The chemical requirements for the induction of xylem cell differentiation are relatively simple. Although a typical culture medium is usually employed, the minimal chemical components required for xylogenesis in excised parenchyma tissue are an auxin, a cytokinin, and a carbohydrate. Some investigations support the view that ethylene is a hormonal requirement (Roberts and Baba, 1978; Miller and Roberts, 1984). Gibberellic acid may interact with other hormones during the in vitro differentiation of secondary xylem fibers (Aloni, 1982).

The developmental process, from the point of determination to the

fully differentiated xylem cell, has been termed the cytodifferentiation sequence. Xylem cells formed under in vitro conditions exhibit the following stages of development after determination: cell enlargement, secondary wall deposition and lignification, and wall lysis and cell autolysis (Roberts, 1976). Although xylogenesis has been the subject of innumerable research investigations, no single stage of the cytodifferentiation sequence is fully understood.

Let us briefly review the nomenclature of the water-transporting cells of the xylem tissue. Tracheary elements, which are subdivided into tracheids and vessel elements, are the conducting cells of the xylem. Both cell types are dead at maturity and possess pitted lignified cell walls. The two types differ in that the tracheids have imperforate end walls, whereas the end walls of the vessel elements have various types of openings or

Fig. 7.1. Cytodifferentiation of tracheary elements in cultured explants of Jerusalem artichoke (*Helianthus tuberosus*) tuber. (a) Tuber cells are shown prior to culture. (b) Cell division occurs following 24 hr of culture. (c) Cytodifferentiation is initiated within 48 hr of culture. (d,e) Safranin O-stained xylem cells show unique secondary wall thickenings. (f, g) Immature nucleate xylem cells are stained with Feulgen reagent and photographed with phase contrast optics. (Courtesy of Dodds and Phillips, 1977.)



perforations. Although tracheids have solid end walls, water and dissolved substances are conducted through the pits in their lateral walls. A longitudinal column of vessel elements, interconnected end to end by their perforation plates, is known as a vessel (Esau, 1977). Cells differentiated in tissue cultures include vessel elements or wound vessel members (Roberts and Fosket, 1962), tracheids, and cells that resemble xylem fibers. Information on the relationship between xylem structure and the ascent of sap can be found in Zimmermann's (1983) monograph.

The classic studies on vascular regeneration around wounded stem internodes should be mentioned because the results of these experiments formed the basis for subsequent in vitro work. The central role of auxin as a limiting hormone in xylogenesis was discovered by Jacobs (1952) as a result of his studies on the formation of wound xylem bridging the severed vascular bundles of *Coleus* stems. Sussex and his colleagues (1972) came to a similar conclusion concerning the role of auxin in xylem formation in wounded tobacco (*Nicotiana*) stems. In addition, vascular regeneration following wounding in pea (*Pisum sativum* L.) roots has been the subject of several recent studies (Robbertse and McCully, 1979; Hammersley and McCully, 1980; Rana and Gahan, 1981; Hardham and McCully, 1982a,b; Rana and Gahan, 1983).

Xylogenesis has been studied in two types of callus: wound callus still attached to the original primary explant and explants derived from a stock that has experienced numerous subcultures and is devoid of any of the original parental tissue (Gresshoff, 1978). The pattern of xylogenesis varies considerably from one culture to another and presumably is governed by several factors. Xylem formation in explants of lettuce pith depends, to some extent, on the combination of auxin and cytokinin present in the medium (Dalessandro and Roberts, 1971). In callus cultures vascular nodules consisting of xylem, phloem, and a cambial zone may be present. The arrangement and cellular composition of vascular nodules can be altered by varying the relative amounts of auxin and sucrose in the medium (Wetmore and Rier, 1963; Jeffs and Northcote, 1966; Fadia and Mehta, 1973; Haddon and Northcote, 1975). Sachs and Cohen (1982) have induced vessel differentiation in the form of rings by certain wound and graft techniques. Although the control of patterned vascular differentiation may be regulated by auxin flux (Sachs, 1981; Sachs and Cohen, 1982), alternative hypotheses have been offered (Wodzicki, Wodzicki, and Zajaczkowski, 1979; Mitchison, 1981). It is beyond the scope of this introduction to discuss the various physical factors that influence xylem differentiation under in vitro conditions (Roberts, 1983).

Whether cell division is a prerequisite for the programming of differentiation is a subject of considerable interest to developmental botanists, and, at this time, the question remains unresolved (Dodds, 1981) Studies have shown that tracheary element differentiation is closely related to cell cycling activity, although some cells apparently differentiate directly without any obvious expression of mitotic division. Although experiments with tuber explants of *Helianthus tuberosus* have shown that three mitotic cycles occur prior to xylogenesis (Malawer and Phillips, 1979; Phillips, 1981a), this requirement is related to the prior development of the parent tuber. Gamma irradiation of tubers, sufficient to prevent both DNA synthesis and mitosis, completely blocked xylem differentiation in explants from mature tubers but not from immature tubers (Phillips, 1981b). Phillips and Arnott (1983) have reviewed cell differentiation in this system. Evidence gained by the use of inhibitors also supports the concept that DNA synthesis is related to this differentiation process (Shininger, 1979). On the other hand, xylem differentiation can occur directly without the progenitor cell exhibiting mitosis. This was demonstrated by the direct formation of tracheary elements from isolated *Zinnia* leaf-mesophyll cells (Kohlenbach and Schmidt, 1975). The *Zinnia* mesophyll system consists of two different cell populations; some of the isolated cells differentiate directly, whereas others require cell cycle activity (Fukuda and Komamine, 1981a,b). Although the time of arrest in the G1 stage of the cell cycle may be the factor that separates the two populations (Dodds, 1981), another hypothesis was given by Fukuda and Komamine (1982). Inhibitor studies with caffeine and colchicine have provided evidence that wound xylem formation in *Pisum* roots is also a direct differentiation process (Hardham and McCully, 1982b)

Additional information on various aspects of xylem differentiation can be found in the monographs edited by Barnett (1981) and Baas (1982).

The purpose of this experiment is to induce the formation of xylem cells in an explant of parenchyma tissue devoid of any preexisting vascular tissue. After varying intervals of time, the explants will be cleared and stained with safranin O. Microscopic observations will be made on the arrangement and morphology of the newly formed xylem cells.

LIST OF MATERIALS

Sterilization mode: C, chemical; O, oven; A, autoclave

C head of cos or romaine lettuce (*Lactuca sativa* L. cv. Romana). Select the largest head available on the basis of basal diameter. Purchase

the head at the time it is uncrated, i.e., before the market and "freshens" it.

paring knife

300 cm^3 aqueous solution (10% v/v) commercial bleach conta a final concentration of approximately 0.5% (v/v) NaOCl

O 600-cm^3 beakers (four); foil wrapped

O 9-cm Petri dishes (five); foil wrapped

O stainless steel cork borer (no. 2) containing a metal rod and prep as in the previous experiments

O stainless steel forceps enclosed in a test tube as previously descr

O culture tubes, 21 × 70 mm (15-cm^3 capacity) recommended (

A 9-cm Petri dishes, each containing two sheets Whatman No. 1 f paper (five)

A 125-cm^3 Erlenmeyer flasks, each containing 100 cm^3 DDH_2O

A 300 cm^3 of MS xylogenic medium supplemented with sucrose w/v) and agar (0.8% w/v). Prepare 3 × 100-cm^3 aliquots in cm^3 Erlenmeyer flasks.

A 9-cm Petri dish equipped as explant cutting guide as describe Chapter 5

C scalpel. Bard-Parker no. 7 surgical knife handle with a no. 10 bl is recommended.

ethanol (80% v/v) dip

ethanol (70% v/v) in plastic squeeze bottle

methanol lamp

interval timer

heavy-duty aluminum foil (one roll)

glass vials (15 × 45 mm) equipped with cork stoppers for clear and staining the explants (30)

aqueous solution NaOH (4% w/v)

aqueous solution safranin O (0.04% w/v)

1 N HCl

glycerol

light microscope (100× magnification)

dissecting microscope or hand lens

dissecting needles

microscope slides; cover slips

incubator or drying oven (50°C)

incubator (25°C)

syringe (2-cm^3 capacity)

PROCEDURE

Preparation of a xylogenic medium. Follow the procedure outlined in the appendix of Chapter 4 for the preparation of 1 liter of MS medium for callus initiation. Since this medium contains both an auxin and a cytokinin, it will induce xylogenesis as well as callus formation in the lettuce pith explants. A typical core yields about 40–50 explants. It is suggested that the student prepare to culture 30 of these explants. Each tube will contain about 10 cm^3 of medium, and therefore it is necessary to prepare 300 cm^3 of nutrient medium. This mixture is prepared in 100-cm^3 aliquots in 250-cm^3 Erlenmeyer flasks.

The instructor may desire to assign each student a different combination of auxin and cytokinin. The relative effectiveness of different cytokinins on inducing xylogenesis may be compared. Each student could use the same auxin stimulus (IAA, 10 mg/l), but choose a different cytokinin (Table 7.1).

An extract of fresh seasonal fruit can be tested for the presence of natural cytokinins. If endogenous cytokinins are found, then xylogenesis should occur in the absence of any additional cytokinin supplement in the medium. The formation of tracheary elements in lettuce pith explants has been used as a biossay system for cytokinins (Banko, Roberts, and Boe, 1976). The student is encouraged to develop a suitable procedure for the preparation of a fruit extract to be incorporated into the MS medium. (Note: see the procedure for the preparation of coconut milk, chap. 4.) In a similar manner, possible auxin activity can be tested by using a standard cytokinin stimulus. Some of the herbicide auxins could be examined, as well as natural plant extracts.

Table 7.1. *Cytokinins for the in vitro induction of xylogenesis*

Chemical or preparation	Suggested concentration range, mg/l
6-furfurylaminopurine (kinetin)	0.1–0.5
6-benzylaminopurine (BAP)	0.1–0.5
zeatin	0.01–0.1
isopentenylaminopurine (IPA)	0.01–0.1
adenine sulfate	50–100
coconut milk[a]	10–20% (v/v)

[a] See Chapter 4.

Culture procedure

Because the steps involved in this experiment are basically the same as the procedure outlined in Chapter 5 for the initiation of callus, only an outline of the steps will be included in this section. The student is requested to review the detailed procedure for the preparation of carrot explants given in Chapter 5 prior to starting the present experiment.

1. Remove the leaves from the head of lettuce, and with a paring knife trim the lateral sides of the basal core until it is smooth. Holding the core with its axis parallel to the bench, slice perpendicular to the axis about 5–10 mm from the basal end. Trim the apical portion by making a second slice perpendicular to the axis about 20 mm from the apex. Assuming a core of medium size, the resulting truncated cone should yield four or five cylindrical borings of parenchyma tissue. Rinse the core with tap water. All subsequent steps must be conducted by aseptic procedures.

2. After surface sterilizing the working area within the transfer chamber with ethanol (70% v/v), surface sterilize the lettuce core with the hypochlorite solution. Remove traces of the hypochlorite solution by passing the core through three successive rinses of DDH_2O.

3. Prepare cylindrical borings, and float the borings in a Petri dish containing DDH_2O.

4. Slice the borings into segments approximately 2.5–3.0 mm in length, and transfer these explants to a Petri dish containing DDH_2O (first rinse).

5. Transfer the explants from the first rinse to a second DDH_2O rinse.

6. Blot each explant on Whatman No. 1 filter paper, and immediately transfer the explant to the surface of the xylogenic medium (one explant per culture tube).

7. Arrange the culture tubes upright in storage jars, and wrap the jars with aluminum foil. Store the cultured explants in an incubator set at 25°C.

RESULTS

The minimum time for xylem differentiation in explants of lettuce pith is about 4 days. In order to terminate the experiment, remove the explants from the culture tubes after 6–8 days of incubation and place them singly in small glass vials. Add about 2 cm^3 aqueous solution of NaOH (4% w/v), stopper the vials, and place them in a 50°C oven overnight.

This heat treatment cleats and softens the tissue. Carefully remove the NaOH solution with a syringe, add about 2 cm^3 aqueous solution of safranin O (0.04% w/v), and return the vials to the 50°C oven. After 30 min remove the dye solution from the vials, fill the vials with 1 N HCl, and return them tc the same oven for about 1 hr. The acid destains the parenchyma cells, but the lignified xylem cells retain the red dye. Remove the discolored HCl from the vials with a syringe, and add a fresh change of HCl for an additional hour. Remove the HCl, and add glycerol to cover the explants. Immersed in glycerol, the explants may be stored indefinitely.

Place one of the explants on a microscope slide, and orient it with the basal end uppermost. The basal end, in this sense, refers to the region of the explant in direct contact with the surface of the medium. Examine it with the low power of a dissection microscope or with a hand lens. Describe the arrangement of the newly formed xylem within the explant. Prepare several glass dissection needles from small-diameter rods of soft glass in the flame of the Bunsen burner. With the aid of a pair of dissection needles, pick out a small fragment of tissue containing clumps or strands of tracheary elements. Transfer the tissue fragment to a clean slide and add a cover glass. Press the center of the cover glass with the tip of a pencil until the tissue is flattened to about 0.5 mm in thickness. Examine the squashed material with a compound microscope (100 × magnification), and describe the appearance of the individual tracheary elements.

A technique for calculating the numbers of xylem cells in an explant is given in the procedure section of Chapter 17.

QUESTIONS FOR DISCUSSION

1. What are some advantages of employing explants consisting entirely of parenchyma cells?
2. Adenine sulfate acts as a ctyokinin in stimulating xylogenesis at concentrations much higher than those of the other cytokinins. Can you offer a possible explanation?
3. How would you define a wound vessel member? a vascular nodule?
4. The pattern of xylem formation in cultured explants is related, to some extent, to the growth regulators used in the medium. Can you offer a possible explanation for this observation?
5. What is the reason for the interest in a possible relationship between cell cycling activity and xylem cell differentiation?
6. Research workers have experienced difficulty in stimulating xylogenesis in cell suspension cultures. Usually differentiation in these cultures does not

occur until the cellular aggregates reach a certain minimal size. Discuss the possible reasons for this observation.

APPENDIX

Alternative plant material. If cos lettuce is unavailable, a variety of other plant material will yield suitable explants for the induction of xylem cell differentiation. Explants from tubers of Jerusalem artichoke (*Helianthus tuberosus*) have been used in numerous studies on xylogenesis. These tubers, which may be marketed in the United States as "sunchokes," are available in the autumn. Soybean (*Glycine max*) cotyledon produces callus containing xylem cells. After surface sterilization of the seeds, soak the seeds overnight in the final DDH_2O rinse, and then slice the softened cotyledons into explants. Xylem cells are formed in callus produced by explants of carrot (*Daucus carota*) taproot. Other suitable vegetables include the storage root of sweet potato (*Ipomea batatas*), the fleshy hypocotyl of radish (*Raphanus sativus*), and the storage root of turnip (*Brassica rapa*). Cultures of the juice vesicle and albedo tissue of *Citrus* form callus containing xylem cells (Kulshrestha, Chauhan, and Roberts, 1982).

Microtubules and xylogenesis. Cytoplasmic microtubules, adjacent to the plasmalemma, are clustered over the developing secondary wall and oriented parallel to the cellulose microfibrils (Hepler, 1982). A simple experiment demonstrates the participation of microtubules in the determination of wall patttern. The alkaloid colchicine specifically binds to protein (tubulin) molecules. The inactivation of this structural protein blocks microtubule polymerization and the formation of the spindle apparatus. The presence of colchicine in the xylogenic medium at the time of explant isolation almost completely blocks xylem formation. This observation suggests that cell division (i.e., involving spindle formation) is a prerequisite for the programming of differentiation. On the other hand, if the explant receives colchicine after the onset of differentiation and at the time of wall deposition, then the pattern of secondary bands is completely lost (Hepler and Fosket, 1971). The student should be aware that colchicine is *highly poisonous*. The dry powder is especially dangerous to the eyes, and it has been reported to cause blindness. *Experiment:* Place explants of lettuce pith on a xylogenic MS medium and, at the same time, culture some explants on an identical medium except for the addition of colchicine (0.04% w/v). After 4 days of culture, transfer a few of the explants that were on the control MS medium to vessels

containing the same medium supplemented with colchicine. After an additional 3 days of culture, clear and stain all of the explants. Note the wavy pattern of secondary wall banding in the explants that received the inhibitor during xylogenesis. Did the explants receiving colchicine during the entire 7 days produce as many xylem cells as the explants receiving the inhibitor for only 3 days?

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8

Root cultures

Liquid media have been employed in tissue culture research mainly in the culture of excised roots and cell suspension (see chap. 9). In addition, various kinds of microcultures, e.g., hanging-drop preparations and protoplast cultures, are special applications of liquid media. Aside from experiments on the formation and development of embryoids, relatively little has been done on the cultivation of organized plant structures other than roots in liquid media. The use of a liquid medium has some advantages over the use of nutrients in an agar-solidified matrix. When a callus is grown on an agar medium, diffusion gradients of nutrients and gases within the callus will lead to modifications in growth and metabolism. The agar itself releases contaminants to the culture medium, and metabolites secreted by the growing callus accumulate in the agar matrix.

One consideration given to plant material growing in a liquid medium is the availability of oxygen and the extent to which the isolated cells or organs require agitation or forced aeration. In regard to root cultures, the importance of oxygenation is unclear. Although Street (1957, 1969) has stated that the availability of oxygen is not a limiting factor in tomato root cultures, this view disagrees with the findings of Said and Murashige (1979). These workers found that continuous and gentle agitation of tomato root cultures resulted in a doubling of root elongation compared to root growth in stationary cultures, and the production and elongation of lateral roots were considerably improved.

There are certain advantages in achieving the continuous culture of an isolated root of a plant. This technique provides information on the nutritional requirements of the root, i.e., removed from the interchange of compounds with other plant organs. We have a detailed account of the nutritional requirements for isolated tomato roots (Street, 1957). In addition to nutritional studies, root cultures from herbaceous species have provided experimental material for investigations on lateral root and bud formation, initiation of cambial activity, and nodulation (see

Torrey, 1965). Another advantage of employing sterile cultures is the elimination of the complicating effects of microorganisms. Root clones have a rapid growth rate, and there are no difficulties in multiplying the clone in order to yield any desired quantity of plant material (Butcher and Street, 1964).

A question has been raised about the relationship between cultured excised roots and similar roots produced by an intact plant. Although excised roots and "intact" roots are alike in many anatomical and metabolic ways, certain differences have been reported. Excised roots gradually lose the capability of forming secondary vascular tissues during culture. Cultured tomato roots, in contrast to seedling roots of the same plant, fail to show a normal geotropic response (Butcher and Street, 1964). In addition, the biochemical composition of cultured roots may differ significantly from that of seedling roots (Abbott, 1963). Studies with microbial symbionts associated with roots have suggested that cultured roots may differ in some respects from the roots of axenically grown seedlings (Torrey, 1978). In spite of these differences, research results obtained from root cultures are relevant to the physiological activity of the roots of higher plants; for example, the production of alkaloids, anabasine and nicotine, continues undiminished in *Nicotiana* root cultures (Solt, Dawson, and Christman, 1960). Although *Solanum* callus synthesizes only traces of glycoalkaloid steroids, the rootlets contain 5.2% (Staba, 1982). Root cultures may become important in the future in the commercial production of useful compounds.

An interesting root culture technique was devised to study the infection of legume roots with *Rhizobium* (Raggio and Raggio, 1956; Raggio, Raggio, and Torrey, 1957). The excised roots were provided with organic nutrients via the basal end of the root inserted in a medium contained in a small vial. The apical portion grew into an inorganic medium in a Petri dish. This arrangement simulated in vivo conditions, i.e., organic metabolites normally enter the root base from the shoot system, whereas minerals are mined from the soil by the tip region. Since *Rhizobium* infects the root via root hairs, the inorganic medium was inoculated with the microorganism. Isolated roots from *Phaseolus vulgaris* and *Glycine max* were used in these studies, and later the method was improved by Cartwright (1967). Torrey (1978) reviewed the use of this technique, as well as other symbiosis studies involving root cultures.

The first successful organ culture, i.e., potentially unlimited growth of the isolated organ, was reported by White (1934) with excised tomato roots. Several modifications of White's medium are currently employed

in plant tissue culture studies (Thomas and Davey, 1975; Said and Murashige, 1979). After the initial experiments with tomato, the excised roots of numerous herbaceous species were placed in culture (Butcher and Street, 1964). Less success has been achieved in starting root cultures from woody plants, although there are reports on cultures from *Acacia* sp. (Bonner, 1942), *Robinia* sp. (Seeliger, 1956), *Acer rubrum* L. (Bachelard and Stowe, 1963), and *Comptonia* sp. (Goforth and Torrey, 1977). Also the roots of several species of gymnosperms have been cultured (Brown and Sommer, 1975). In some cases, the minimum growth requirements were met with the essential minerals, a carbon source, a vitamin supplement, and a few amino acids. Some responded favorably to the addition of auxin and other growth regulators. Inositol is an effective growth stimulant for some isolated roots, and this cyclitol plays a role in secondary vascular tissue formation in excised roots of radish (*Raphanus*) (Loomis and Torrey, 1964; Torrey and Loomis, 1967).

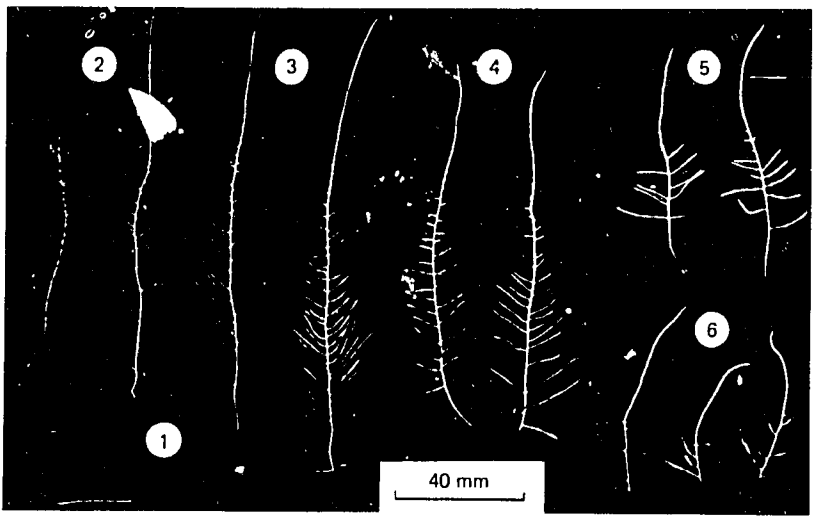
The technique employed to initiate and subculture roots requires some explanation. Root tips approximately 10 mm in length are removed from young seedlings produced during the axenic germination of seeds, and these apical tips are transferred to an aqueous culture medium. After about 1 week the primary root produces lateral roots. The main root axis is subdivided into "sectors," and each contains a portion of the main axis plus several lateral branch roots. Each of these sectors is transferred separately to a fresh medium for an additional period of incubation. The lateral roots subsequently produce laterals themselves. Each of these sector cultures provides the investigator with a constant supply of lateral root tips for experiments, as well as a source of material for the propagation of the clone. This technique can be used only when the excised root produces laterals in some sequential order. In some species this does not occur.

Some general comments can be made about the nutrition of cultured roots. A marked improvement over White's medium is the substitution of a chelated form of iron for $Fe_2(SO_4)_3$, because use of the latter salt leads to an iron deficiency during prolonged culture. During root growth an alkaline shift in pH occurs, which results in the precipitation of $Fe(OH)_3$ (Guinn, 1963) and possibly $Fe_2(PO_4)_3$ (Dalton, Iqbal, and Turner, 1983). The sodium salt of ferric ethylenediamine tetraacetic acid (NaFeEDTA) is generally used as a chelated form of iron. Sucrose is the carbon source of choice, although some monocot roots grow equally well with glucose. A sugar level of 1.5–2.0% (w/v) is sufficient, and higher concentrations may alter root metabolism (Guinn, 1963; Fig. 8.1). Al-

though several amino acids have been tested, most of them inhibit the growth of cultured roots. The vitamin requirements vary slightly for different species, although all species require the addition of thiamine. Cereal roots have a higher auxin requirement in comparison to dicot roots. Street (1957) reported that Petkus II rye roots required either auxin or an auxin precursor for continued growth. The effects of light on the growth of cultured roots, however, appear to vary with the species and the cultural conditions.

In the present experiment, sterile seedling roots obtained from the axenic germination of seeds taken from a fresh tomato fruit will be excised and cultured in a modified White's medium. After approximately 7–10 days of growth, sufficient lateral root development will have occurred to permit the first subculture with sector inocula (Fig. 8.1). Through periodic subcultures the student should maintain the root culture over a period of several weeks. Various experiments can be devised in order to determine the optimum conditions for growth and development of the isolated roots.

Fig. 8.1. Excised tomato (*Lycopersicon esculentum*) roots cultured 7 days at 27°C in White's medium containing sucrose at a concentration of (1) 0.5%, (2) 1.0%, (3) 1.5%, (4) 2.0%, (5) 3.0%, and (6) 4.0%. Note the formation of lateral roots from the main root axis. In order to subculture roots, the root is cut into sectors. Each sector, transferred to a fresh medium, contains a portion of the main root axis plus several lateral roots. (Courtesy of H. E. Street.)



LIST OF MATERIALS

Sterilization mode: C, chemical; O, oven; A, autoclave

C fresh ripe tomato (*Lycopersicon esculentum*) fruit with no surface blemishes or breaks in the skin

O stainless steel forceps

C scalpel. Bard-Parker no. 7 surgical knife handle equipped with a no. 10 blade is recommended.

A 9-cm Petri dishes, each containing two sheets Whatman No. 1 filter paper (five)

A 125-cm^3 Erlenmeyer flask containing 100 cm^3 DDH_2O (one)

A 10-cm^3 pipette; cotton plugged and enclosed completely in heavy wrapping paper

A 125-cm^3 Erlenmeyer flasks, each containing 25 cm^3 modified White's medium as given in the formulation at the end of the book (five)

A paper toweling; several sheets enclosed in mailing envelope

ethanol (80% v/v) dip

ethanol (70% v/v) in plastic squeeze bottle

methanol lamp

graph paper (one sheet)

incubator (25°C)

iridectomy scissors (optional)

orbital platform shaker (optional)

PROCEDURE

Preparation of White's medium (see formulations of tissue culture media at the end of the book). In general, use the same procedure outlined in Chapter 4 for the preparation of the MS medium, i.e., first prepare stock solutions of iron, micronutrients, and vitamins. Because of the small amount of molybdenum involved, weigh 10 mg MoO_3, dissolve it, and add DDH_2O for a final volume of 1,000 cm^3. Pipette 1 cm^3 to deliver 0.01 mg MoO_3 to the micronutrient stock.

White's (1963) formulation required $Fe_2(SO_4)_3$. Because ferric sulfate precipitates easily from solution, a chelated form of iron is more desirable. The concentration of iron employed in the MS medium can be used satisfactorily in White's medium. Prepare and use the stock as directed in Chapter 4.

The vitamins are dissolved in DDH_2O in the order indicated, and a final stock volume of 100 cm^3 is prepared.

Add approximately 400 cm^3 DDH_2O to a 1 liter beaker. Weigh and dissolve each of the macronutrient salts given in the table of formulations. From each of the stock solutions add by pipette to the macronutrient solution: 5 cm^3 iron, 10 cm^3 micronutrients, and 1 cm^3 vitamins. The final medium is adjusted to pH 5.5, and sucrose is supplied at a concentration of 20,000 mg/l of final medium. (*Note:* A possibility exists of some degradation of the B vitamins during autoclaving.)

Culture procedure

The working area of the transfer chamber is lined with paper toweling, previously sterilized by autoclave. The folded toweling is enclosed in a heavy paper mailing envelope for sterilization. Wash the tomato fruit with tap water. Surface sterilize the skin of the fruit with ethanol (70% v/v) in the transfer chamber. Using sterile instruments, cut the skin and slice the fruit into four sectors. Pull the sectors apart exposing the seeds. Remove the seeds with sterile forceps, and carefully remove the fruit pulp around each seed. Place approximately 5 seeds in each of the Petri dishes lined with Whatman No. 1 filter paper. Moisten the filter paper with about 8–10 cm^3 of sterile DDH_2O delivered by pipette. Wrap the dishes in aluminum foil, and place them in the incubator (25°C). The seeds will germinate within a few days. The Petri dishes must be checked frequently for signs of drying of the filter paper. If necessary, a few drops of sterile water may be added. Root tips approximately 10 mm in length are excised with a scalpel or iridectomy scissors and transferred to the flasks containing White's medium. White (1963) suggests that the root tips be transferred individually, i.e., one per culture flask. After about 1 week, sufficient lateral root development will be evident, and sector inocula (Fig. 8.1) can be removed and subcultured to a fresh medium. The student should perform weekly transfers and attempt to maintain the tomato root culture over a period of several weeks.

RESULTS

Although cultured roots are difficult to measure in situ during culture, an attempt will be made to approximate the linear growth rate. Daily measurements can be made on a single culture, within the flask, without exposure and risk of contamination. Place the flask on a sheet of graph paper, and align the root with the markings on the paper. Compare the growth rates of root cultures that have been stationary to the growth rates of other cultures that received agitation on a shaker. An interesting

comparison can also be made on roots cultured in the dark versus othe roots that received illumination. The incubation temperature will alsc influence the growth rate. The growth rates of tomato roots culturec under various conditions have been reported by Boll (1965).

QUESTIONS FOR DISCUSSION

1. What are some advantages of using an aqueous medium in comparison to an agar-solidified medium? Can you think of any advantages that were not mentioned in this chapter?
2. In addition to providing a source of oxygen, what are some other possible effects of agitating cultured plant cells, cell aggregates, or organs?
3. What are the nutritional requirements for the culture of isolated tomato roots? Do all isolated roots have approximately the same nutritional requirements?

APPENDIX

Proliferation of root hairs in culture. An unusual technique has been developed by Hermina and Reporter (1977) for the production of root hairs in a culture of epidermal cells isolated from the hypocotyl of soybean (*Glycine max*) seedlings. This procedure was developed in order to study the symbiosis between *Rhizobium* and soybean cells under in vitro conditions. After 3 days of axenic germination (dark, 26°C) of the seeds on moist filter paper in Petri dishes, excise explants 3–4 mm in length from the hypocotyl. Arrange the explants in a circle in a Petri dish containing a modified PRL-4 medium (Gamborg and Everleigh, 1968) with agar (1% w/v). The latter medium is identical with the B5 medium given in the formulation table except for the following: $NaH_2PO_4 \cdot H_2O$ (90 mg/l), Na_2HPO_4 (30 mg/l), KCl (300 mg/l), $(NH_4)_2SO_4$ (200 mg/l), and casein hydrolysate enzymatic (2 mg/l; available from ICN Nutritional Biochemicals, P.O. Box 28050, Cleveland, OH 44128, U.S.A.; order no. 101290). The pH of the medium is adjusted to 6.2. Unless otherwise stated the PRL-4 and B5 media used in this experiment are not supplemented with plant growth regulators. A droplet (0.05 cm^3) of 2,4-D solution (10^{-5}M) is placed in the center of the dish containing the explants. The dish is enclosed with plastic film, but not sealed, and the culture is incubated at 26°C. After 8–10 days of culture, distorted growth will be evident by the protrusion of the central region of vascular tissue from the ends of the explants. With the aid of two pairs of forceps, strip the epidermal and cortical layers from the explants. Suspend these tissues in 4–5 cm^3 liquid B5 medium, and pour the tissue suspension on the

surface of a Petri dish containing agar-solidified B5 medium. Incubate the dish overnight at 26°C. The tissue suspension is then transferred to a nylon mesh (200-μm pore size) and washed with B5 medium for the removal of most of the cortical cells. The remaining epidermal and residual cortical cells are removed from the mesh and suspended in approximately 4–5 cm^3 of liquid B5 medium. The cell suspension is then poured onto the surface of a Petri dish containing agar-solidified B5 medium. The Petri dish is enclosed with plastic film, although some ventilation is allowed for gas exchange. After 3 days of incubation (26°C) the liquid medium is removed by inclining the dish and withdrawing the liquid with a Pasteur pipette. The cultured cells remain attached to the surface of the agar. The spent B5 medium is replaced with 4–5 cm^3 PRL-4 medium supplemented with kinetin and 2,4-D. Both growth regulators are present at a concentration of 1.0 μM. Although root hairs may be evident at various stages of the procedure, the hormone treatment at this step accelerates their development. Soybean epidermal root cells have a characteristic kidney shape, and root hair formation occurs on the concave side of the cells. It is advisable to replace the liquid medium with a fresh aliquot every 7–8 days. An inoculum of the culture can be transferred to a 50-cm^3 Erlenmeyer flask containing 6–7 cm^3 of liquid PRL-4 medium containing the same concentrations of kinetin and 2,4-D as the agar cultures. The cell suspension is cultured on an orbital shaker (100 rpm; 26°C).

Salinity and root growth. Repeat the experiment on the culture of tomato roots as outlined in this chapter except the student should prepare a series of culture flasks numbered 1 through 6. Flask no. 1 will contain White's medium as a control. Flasks 2–6 will contain White's medium supplemented with a range of concentrations of NaCl (0.1, 0.5, 1.0, 1.5, and 2.0% w/v). Approximate the linear growth rate, on a daily basis, of the tomato roots cultured in each of the flasks. Explain the results. How would you devise an experiment to determine whether the effect of NaCl on root growth was due to sodium toxicity or to osmotic potential?

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9

Cell suspension cultures

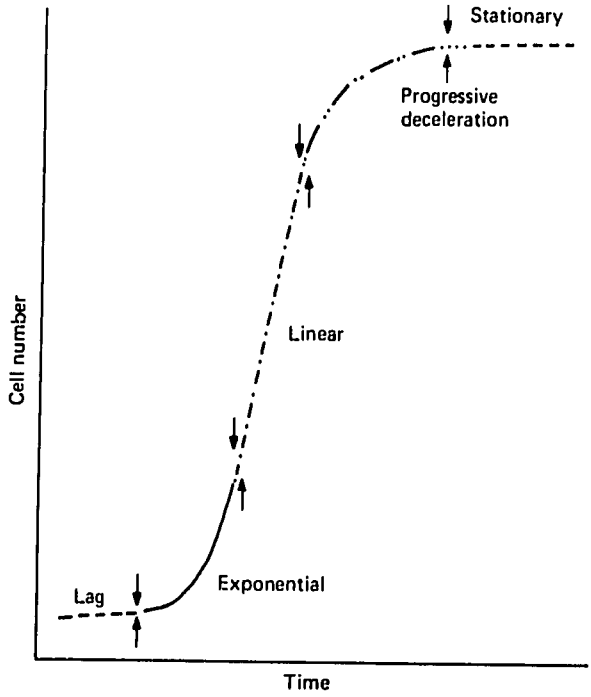
According to King (1980) the term "suspension culture" has no clear-cut biological definition, and these tissue culture systems are evidently more than simply aggregates of cells suspended in a liquid medium. A suspension culture originates with a "random critical event" occurring during the early exposure of the plant cells to the liquid medium. Cells undergoing this transition in metabolism and growth rate produce a "cell line." Some of the characteristics of cell lines include (a) a high degree of cell separation, (b) homogeneous cell morphology, (c) distinct nuclei and dense cytoplasm, (d) starch granules, (e) relatively few tracheary elements, (f) doubling times of 24–72 hr, (g) loss of totipotency, (h) hormone habituation, and (i) increased ploidy levels (King, 1980). Suspension cultures vary considerably in the expression of these and other cell line characteristics, and consequently these systems remain an ill-defined group.

Cell suspension cultures are generally initiated by transferring fragments of undifferentiated callus to a liquid medium, which is then agitated during the culture period. Although a longer time is required, suspension cultures can be started by inoculating the liquid medium with an explant of differentiated plant material (e.g., a fragment of hypocotyl or cotyledon). The dividing cells will gradually free themselves from the inoculum because of the swirling action of the liquid. It should be kept in mind, however, that no suspension culture has been shown to be composed entirely of single cells (Butcher and Ingram, 1976). After a short time the culture will be composed of single cells, cellular aggregates of various sizes, residual pieces of the inoculum, and the remains of dead cells. The term "friability" is used to describe the separation of cells following cell division. Formation of a "good suspension" (i.e., a culture consisting of a high percentage of single cells and small clusters of cells) is much more complex than finding the optimum environmental conditions for cell separation (King, 1980). The degree of cell separation of

established cultures already having the characteristics of high friability can be modified by changing the composition of the nutrient medium. Increasing the auxin:cytokinin ratio will, in some cases, produce a more friable culture. On the other hand, some cultures exhibit low friability regardless of cultural conditions (King and Street, 1977). There is no standard procedure that can be recommended for starting cell suspension cultures from callus; the choice of suitable conditions is largely determined by trial and error (King and Street, 1977).

The initiation of a cell suspension culture requires a relatively large amount of callus to serve as the inoculum, for example, approximately 2–3 g for 100 cm^3 (Helgeson, 1979). When the plant material is first placed in the medium, there is an initial lag period prior to any sign of cell division (Fig. 9.1). This is followed by an exponential rise in cell number, and a linear increase in the cell population. There is a gradual deceleration in the division rate. Finally, the cells enter a stationary or nondividing stage. In order to maintain the viability of the culture, the cells should be subcultured early during this stationary phase.

Fig. 9.1. Growth curve of a cell suspension grown under batch conditions relating total cell number per unit volume to time.

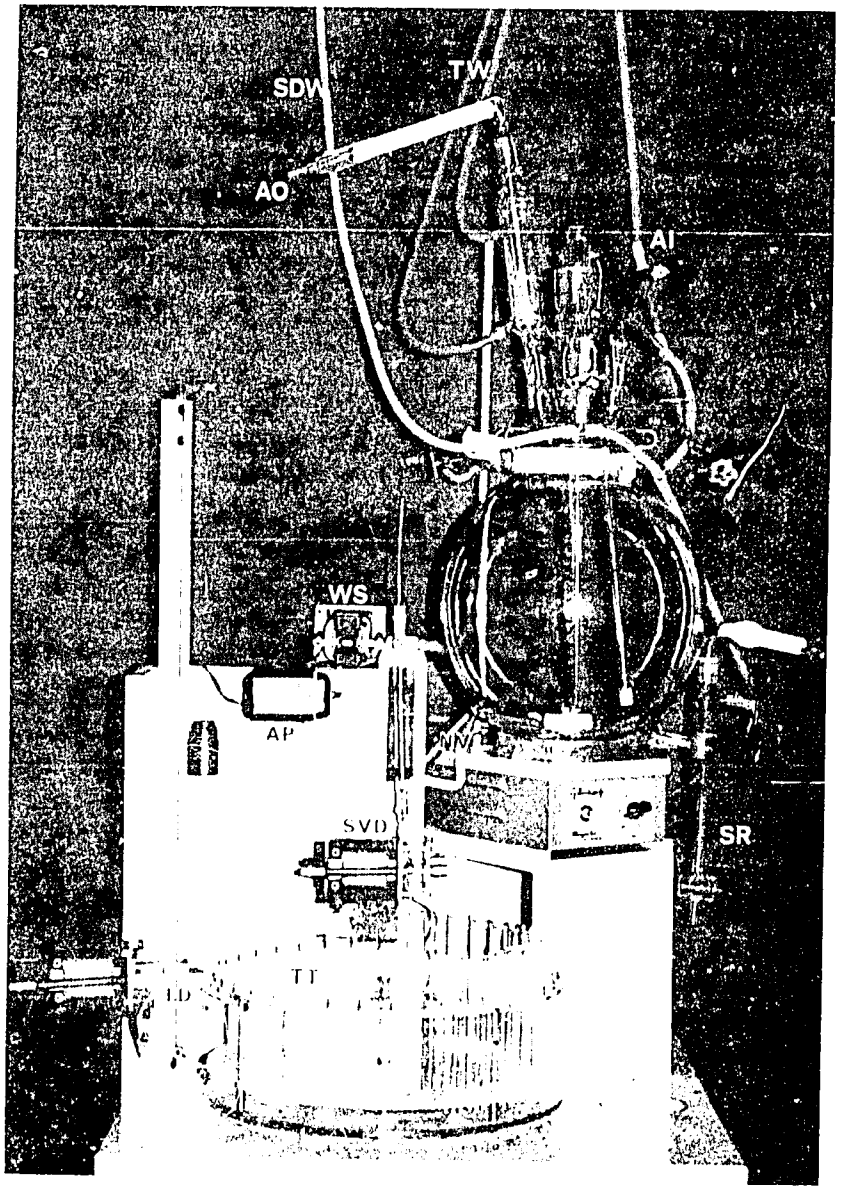


Because cells from different plant material vary in the length of time they remain viable during the stationary phase, it may be prudent to subculture during the period of progressive deceleration. Passage time can be learned only from experience, and a given suspension culture should be subcultured at a time approximating the maximum cell density. For many suspension cultures the maximum cell density is reached within about 18 to 25 days, although the passage time for some extremely active cultures may be as short as 6 to 9 days (Street, 1977). At the time of the first subculture it will be necessary to filter the culture through a nylon net or stainless steel filter to remove the larger cell aggregates and residual inoculum that would clog the orifice of a pipette. A small sample should be withdrawn, and the cell density determined before subculturing. There is a critical cell density below which the culture will not grow; for example, this value is $9–15 \times 10^3$ cells/cm^3 for a clone of sycamore cells (*Acer pseudoplatanus*) (Street, 1977).

Cell suspension cultures must be agitated or subjected to forced aeration, and a platform (orbital) shaker is used for this purpose in most laboratories. The best speed range for cultures in 250-cm^3 Erlenmeyer flasks is 100–120 rpm (Thomas and Davey, 1975). The volume of liquid in relation to the size of the flask is important for adequate aeration (i.e., the liquid medium should occupy about 20% of the total volume of the flask). Other devices for aeration include magnetic stirrers, roller cultures, and Steward's auxophyton. The latter apparatus slowly rotates the cultures in nipple flasks and tumble tubes. Microcultures do not need any device for oxygenation of the nurtured cells.

Some terminology is associated with cell suspension cultures. The present experiment involves the preparation of a batch culture (Fig. 9.2). This is defined as a culture grown in a fixed volume of culture medium. Our experiment is also a closed culture, because all cells are retained, and a continual increase in cell density will occur until the stationary phase is reached. A closed continuous system involves a continuous influx of fresh medium and a withdrawal of spent medium. An open continuous system is similar to the closed in the replenishment of the nutrient medium; in addition, the cells are harvested. Examples of open continuous systems are chemostats and turbidostats. In a chemostat the continuous flow of fresh medium into the system is set at a predetermined rate; this influx of nutrients will largely determine the growth rate of the culture. Cell density is set at some predetermined level in the turbidostat, and fresh medium is added periodically in order to maintain the cell density within the preset limits. Cell density in the turbidostat is determined with a photocell control device.

Fig. 9.2. Assembly for automatic sampling of large-scale batch culture. Key: air inlet (AI); air outlet (AO); air pump (AP); latching device (LD); needle valve for automatic sampling (NV); sterile distilled water (SDW); sample receiver for manual samples (SR); sample volume detector (SVD); turntable (TT); tap water lines (TW); and wash solenoid valve (WS). (Courtesy of N. Everett.)



In the present experiment it is suggested that the callus culture d scribed in Chapter 5 be used as the inoculum for the cell suspensi culture. Carrot callus is an excellent inoculum for a suspension cultur

A detailed account of the nutritional requirements of cell suspensi cultures is beyond the scope of this introduction; however, some speci refinements in the nutritional needs of cultures are discussed in the r view by Ojima and Ohira (1978).

LIST OF MATERIALS

Sterilization mode: C, chemical, O, oven, A, autoclave

actively growing callus cultures, preferably derived from carrot tap root (*Daucus carota* L.) (5–10 culture tubes)

O forceps

C scalpel

A 9-cm Petri dishes, each containing two sheets Whatman No. 1 filte paper (two)

A 125-cm^3 Erlenmeyer flasks, each containing 25 cm^3 liquid MS me dium, 2,4-D (1 $\mu g/cm^3$) and sucrose (750 mg) (five)

A 100-μm pore size nylon mesh filtration cloth equipped with a Hirsl funnel and flask

A syringe equipped with wide-bore cannula, preferably designed tc deliver a preset volume

A Pasteur pipette or 1-cm^3 pipette for sampling culture

ethanol (80% v/v) dip

ethanol (70% v/v) in plastic squeeze bottle

aqueous solution chromium trioxide (8% w/v)

hot plate

Pasteur pipette for sample maceration

Sedgwick-Rafter slide or hemocytometer

depression slide; microscope slide; cover slips

light microscope (100 × magnification)

dissecting microscope

125-cm^3 beaker

ocular micrometer, calibrated (100 ×) for the light microscope (optional)

PROCEDURE

Carrot cell suspension culture. Carrot cell suspension cultures can be initiated on a basal MS medium supplemented with 2,4-D (1.0 mg/l) and

sucrose (Nishi and Sugano, 1970). A suspension culture of carrot does not require an exogenous cytokinin (Nishi and Sugano, 1970), and the presence of auxin as the sole plant growth regulator in the medium may improve the friability of the culture. For the present experiment each 125-cm^3 Erlenmeyer flask will contain 25 cm^3 of basal MS medium, plus 2,4-D (1 $\mu g/cm^3$) and sucrose (750 mg).

The callus resulting from the experiment conducted in Chapter 5 should be removed from the culture tubes with forceps and transferred to a Petri dish containing Whatman No. 1 filter paper. Trim the callus blocks with the scalpel, and use only the young actively growing callus for the inoculum. Each flask should receive an inoculum of about 500–750 mg of callus in order to ensure the initiation of the culture. Brownish callus may be indicative of senescence and should be discarded. Place the inoculated flasks on the shaker and set the speed at 100 rpm. The shaker with the flasks should be placed in an air-conditioned enclosure maintained at 25–27°C. If, during the first few days, the medium appears "milky," this is a sign that contamination occurred during the inoculation. The initial subculture can be performed after 7–10 days, although it is first necessary to filter the culture through an industrial nylon mesh filter in order to remove the residual inoculum and larger clumps of cells. This is somewhat difficult because the cell sizes in carrot suspension cultures range from 50 to 300 μm in diameter (Steward, Mapes, and Smith, 1958). A nylon mesh filter (100 μm pore size), sterilized with a Hirsh funnel, will be satisfactory. The next step is to determine the cell density of the culture. It is impossible to subculture and maintain the culture unless the cell density is within a given range. According to Street (1977), most suspension cultures contain $0.5–2.5 \times 10^5$ cells/cm^3 after dilution with the fresh medium. A sample is taken with a syringe equipped with a wide-bore cannula. Cell counting is best achieved by a closely regulated cell separation with chromium trioxide. Because the acid treatment is highly destructive, it should be long enough to achieve a reasonable degree of cell separation without destroying the sample (Street, 1977). Chromium trioxide is *highly corrosive*. Be particularly careful not to spill the acid on your skin or clothing or to breathe the fumes. Add one volume of the cell suspension to two volumes of chromium trioxide (8% w/v), heat the mixture to 70°C for 2–15 min inside a hood, cool it, and then macerate the sample further by pumping it repeatedly through the orifice of a Pasteur pipette. The macerate is placed in a Sedgwick-Rafter slide or hemocytometer for cell counting. Discard the acid solution with great care, and rinse the Pasteur pipette and slide after use. Additional details on the quantitation of results are given in Chapter 17. The nec-

essary volume of inoculum may be calculated in order to give a final concentration of cells within the minimum density level (i.e., about 0.5–2.5 × 10^5 cells/cm^3).

RESULTS

Remove a 1-cm^3 sample of the cell suspension culture with a sterile Pasteur pipette, and discharge the contents into a small beaker. Place about 0.1 cm^3 of the suspension in a depression slide, and examine the preparation with a dissection microscope. Then examine a droplet of the suspension with the light microscope (100 ×). In what obvious ways do these cultured cells differ from the cells of the primary explant excised from the cambial zone of the carrot root? With the aid of an ocular micrometer, the approximate range of sizes of the cells can be estimated. Some of the cellular details may be enhanced by employing a biological stain for increasing the contrast (see chap. 5).

One of the cell suspension cultures may be sampled daily and the cell density determined. Prepare a plot of the cell number versus time. Does the resulting curve of the growth rate approximate the curve shown in Fig. 9.1?

QUESTIONS FOR DISCUSSION

1. What are some advantages of using an aqueous medium in comparison with an agar-solidified medium? Can you think of any advantages that were not mentioned in this chapter?
2. In addition to providing a source of oxygen, what are some other possible effects of agitating a cultured plant cell or organ?
3. From the time of inoculation, what growth stages are exhibited by a cell suspension culture? Why does the rate of growth resemble an S-shaped curve (i.e., what are the reasons for each of these fluctuations in the growth curve)?
4. Define each of the following terms: batch culture and open culture.

APPENDIX

Viability of cell suspension. A method has been reported for the determination of cell viability in suspension cultures (Towill and Mazur, 1975). Viability, in this instance, refers to the capability of the cells to exhibit cell division. The tetrazolium reagents accept electrons from the electron transport chain of the mitochondria; as a result, these oxidation-reduction indicators are converted to brightly colored formazan precipitates. There is a close positive correlation between the amount of formazan produced

and the percentage of viable cells in the sample. The technique involves the preparation of an aqueous solution of 2,3,5-triphenyltetrazolium chloride (0.8% w/v) dissolved in a mixture of buffer and suspension culture in the ratio of 2 parts buffer to 1 part suspension culture. A sodium phosphate buffer (0.05 M) giving a final pH of 7.5 is recommended. After an 18–20-hr incubation in the dark at room temperature, a red precipitate will be observed in the viable cells. The cells are pelleted by centrifugation and washed once with distilled water, and the dye is extracted with 3 cm^3 ethanol (95% v/v) for 30 min. If cell clumps are present, gentle heating will facilitate the extraction (60°C; 5–15 min). The absorbance is determined in a spectrophotometer (485 nm). *Experiment:* Take a sample of the carrot cell suspension culture that has been subjected to cell counting by Sedgwick-Rafter slide or hemocytometer, and determine the absorbance of the formazan dye produced by the preceding technique. Prepare serial dilutions of the sample, and redetermine the formazan content of each of the dilutions. Do you find a linear relationship between cell numbers and dye production in the series of dilutions?

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10

Micropropagation with shoot-apex cultures

Early experiments with shoot-apex cultures involved the degree of autonomy shown by this unit and the morphogenetic consequences of its isolation. White (1933) made one of the first attempts to culture the shoot tip using *Stellaria media*. Later, Loo (1945) succeeded in culturing stem tips, some 5 mm in length, excised from *Asparagus officinalis*. The subculture of the cladophylls, formed on the cultured asparagus shoots, resulted in the formation of plantlets. From these early studies the concept was developed that ferns and angiosperms differ in the degree of autonomy of their isolated shoot apices. Isolated fern apices regenerated plants on a simple defined medium (Wetmore and Morel, 1949; Wetmore, 1953), whereas the cultural requirements for angiosperms were more complicated. Ball (1946) found that root initiation and plantlet formation occurred if the tip explant included a minimum of three leaf primordia and subjacent stem tissue. Smith and Murashige (1970), however, succeeded in culturing apical meristems devoid of any leaf primordia from two species of *Nicotiana*, *Daucus carota* L., *Tropaeolum majus* L., and *Coleus blumei* Benth. The explants, consisting only of meristematic dome tissue, produced complete plants following culture on a defined medium.

Because of the extensive application of shoot-tip cultures in horticulture and plant pathology, there has been a flagrant misuse of the botanical nomenclature (Murashige, 1974). The terms "meristem culture," "meristemming," and "mericlones" have been used for the culture of relatively large stem tips; for example, orchid growers often use sections as long as 5–10 mm (Murashige, 1974). Cutter (1965) clearly distinguishes the apical meristem from the shoot apex. The apical meristem refers only to the region of the shoot apex lying distal to the youngest leaf primordium, whereas the shoot apex refers to the apical meristem plus a few subjacent leaf primordia. The apical meristem explants employed by Smith and Murashige (1970) were about 80 μm in height.

Such minute explants are extremely difficult to excise, have a very low survival rate, and are impractical for the in vitro propagation of plants. Apical meristem cultures, however, are important in the development of pathogen-free stock (see chap. 18).

The application of shoot-apex cultures for the clonal multiplication of plants was first realized by Morel (1960) in his studies on the propagation of the orchid *Cymbidium*, and modifications of his technique are currently used for the commercial production of orchids. The rapid progress in micropropagation techniques using multiple shoot formation was due largely to the contributions of Murashige. Murashige (1974) subdivided the procedure into three stages: stage I, establishment of the aseptic culture; stage II, multiplication of propagula by repeated subcultures on a multiplication medium; and stage III, preparation of the plantlets for establishment in the soil. The entire method of micropropagation with shoot tips is based on the cytokinin-induced outgrowth of bud primordia, each of which produces a miniature shoot. Once a cluster of shoots has been formed, it can be subdivided into smaller clumps of shoots, transferred to a fresh medium, and the process is repeated. The rates of micropropagation vary greatly from species to species, but it is often possible to produce several million plants in the period of a year starting from a single isolated shoot tip (Thomas, 1981; Wilkins and Dodds, 1983).

Factors influencing stage I include the choice of a suitable explant, the composition of the medium, and the environmental conditions. Shoot tips and buds excised from healthy and actively growing herbaceous plants are generally ideal material for multiple shoot production. The larger the size of the tip explant, the more rapid the growth and the greater the rates of survival (Hussey, 1983). A procedure that does not involve a callus phase is preferable, because the genetic instability of callus leads to a high degree of genetically aberrant plants. Although some groups of plants have unique nutritional requirements, the MS formulation is satisfactory in most cases. The basal medium is supplemented with vitamins, sucrose, and the appropriate hormones. The cultures can be grown on agar or on filter-paper bridges (Goodwin, 1966) over a liquid medium. Light, necessary for photomorphogenesis and chlorophyll biosynthesis, is provided with Gro-Lux and white fluorescent tubes. Murashige (1974) found that many cultures grew best with 1,000 lux for stages I and II, with the light intensity increased to 3,000–10,000 lux for stage III. Photoperiods of 16 hr were optimum for several species (Murashige, 1977), although relatively little research has been done on the effects of light and temperature on micropropagation.

Typically, the same medium and environmental conditions are used for both stages I and II. The choice and concentration of hormones are the most important considerations in preparation of the medium. Cytokinins may be added in the form of kinetin, benzyladenine, IPA, or zeatin. Some of Murashige's multiplication media that are commercially available contain combinations of adenine sulfate plus either kinetin or IPA. The source of exogenous auxin is usually IAA, NAA, or IBA (indole-3-butyric acid). The auxin 2,4-D is unsatisfactory, since it stimulates callus formation and strongly suppresses organogenesis. Gibberellic acid may be required for the culture of some shoot apices. Morel (1975) reported that isolated apices of potato, *Dahlia*, carnation, and *Chrysanthemum* need exogenous GA_3 (0.1 mg/l) for the normal development of the shoot apex and subsequent micropropagation (Fig. 10.1).

Stage III involves the development of a root system, hardening the young plant to moisture stress, increasing resistance to certain pathogens, and conversion of the plants to an autotropic state (Murashige, 1974). Root initiation may be facilitated by adding a low concentration of either NAA or IBA to the medium. The auxin treatment must be limited to a brief period of time. Auxin at this stage of the process may have undesirable side effects, i.e., stimulate callus production and inhibit root elongation (Hu and Wang, 1983). The formation of roots often occurs after transfer to a medium lacking hormones. In fact, the shoots of some species can be rooted by conventional root procedures after removal from the in vitro environment (Debergh and Maene, 1981).

Tissues containing relatively high concentrations of phenolic compounds are difficult to culture. Polyphenolases stimulated by tissue injury will oxidize these phenolic substances to growth-inhibiting, dark-colored compounds. Techniques used to suppress this metabolic sequence include (1) adding antioxidants to the medium, (2) presoaking the explants in antioxidant solutions prior to culture, (3) subculturing to a fresh medium on signs of enzymatic browning, and (4) providing little or no light during the initial period of culture (Hu and Wang, 1983). For example, the brown exudate resulting from polyphenolase activity in *Eucalyptus grandis* cultures was eliminated by the following protocol. Explants were prepared from young stem tissues, and the explants were presoaked in sterile distilled water for 3 hr prior to culture. The explants, leached of phenolic compounds, were then initially incubated in darkness for several days (Durand-Cresswell, Boulay, and Francelet, 198_). Additional information on the elimination of polyphenolase activity can be found in the review by Hu and Wang (1983).

Although the successful micropropagation of many tree crop species

has been achieved using shoot-apex cultures, woody plants pose some unique problems. The greatest success is achieved with bud cultures taken either from shoots in the juvenile growth phase or selected from rejuvenated shoots. Buds taken from mature trees in the adult phase

Fig. 10.1. Micropropagation of potato (*Solanum tuberosum*). (a) Excised shoot tip grows to produce a plantlet. (b) In micropropagating culture multiple shoots are produced by outgrowth of axillary buds. (c) In vitro plantlet transferred to "jiffy" pot. (d) Plantlet derived from shoot tip ready for transfer to field.

(a)

(b)

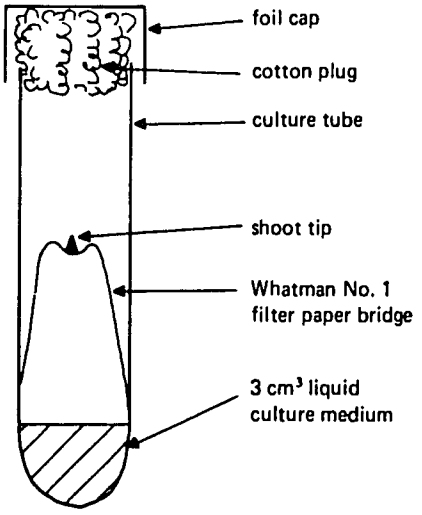
(c)

(d)

have relatively little capacity for micropropagation (Bonga, 1982; Jones, 1983). Shoot-apex cultures of woody plants require several consecutive treatments (Wilkins and Dodds, 1983). The excised bud, with the formation of a rosette of leaves, requires exogenous gibberellin and cytokinin for growth. The explant must then be transferred to another medium lacking exogenous growth regulators in order to promote stem elongation. Finally, the culture must be transferred to a third medium containing exogenous auxin for the initiation of roots (Morel, 1975).

In the following experiment the student will excise and micropropagate some shoot apices from either potato (*Solanum tuberosum*) or geranium (*Pelargonium*). An attempt will be made to propagate whole plants from the excised and cultured shoot apices. The filter-paper bridge technique (Goodwin, 1966) will be employed as shown in Fig. 10.2. The explants are cultured individually in a rimless Pyrex test tube (16 × 150 mm) containing a strip of Whatman No. 1 filter paper (9 × 90 mm) folded in the shape of the letter *M*. The arms of the paper strip are immersed in the liquid medium in the bottom of the tube. The explant, positioned upright in the V of the bridge, is about 10–15 mm from the level of the medium.

Fig. 10.2. Culture tube assembly employed in the culture of shoot apices. The explant is carefully placed in an erect position in the central depression of the filter-paper bridge.



LIST OF MATERIALS

Sterilization mode: C, chemical; O, oven; A, autoclave

shoot-apical material from potato (*Solanum tuberosum*) or geranium (*Pelargonium*)

C scalpel or razor blade for microsurgery. Use a surgeon's scalpel fitted with a no. 11 blade (Shabde-Moses and Murashige, 1979), or prepare microscalpels by flattening the tips of no. 2 insect mounting pins (Smith and Murashige, 1970).

filter-paper bridges prepared from Whatman No. 1 filter paper (9 × 90-mm strips)(12)

A Pyrex test tubes (16 × 150 mm), each containing a filter-paper bridge and 3 cm^3 MS medium supplemented with IBA (2.0 mg/l), BAP (2.0 mg/l), GA_3 (0.1 mg/l), and sucrose (2% w/v). Close the tubes with cotton plugs and foil caps (12).

A 9-cm Petri dishes, each containing two sheets Whatman No. 50 filter paper (five)

A 125-cm^3 Erlenmeyer flasks, each containing 100 cm^3 DDH_2O (eight)

A 5-cm^3 pipette

O forceps

O 250-cm^3 beakers (four)

100 cm^3 aqueous 10% (v/v) solution commercial bleach

ethanol (80% v/v) dip

ethanol (70% v/v) in plastic squeeze bottle

methanol lamp

binocular dissection microscope with external top lighting (50 ×)

plant growth chamber (16/8-hr photoperiods; 1,000 lux illumination; 27°C; 70% relative humidity)

PROCEDURE

1. Insert a filter-paper bridge folded in the shape of the letter *M* into each culture tube. Add by pipette 3 cm^3 of MS medium (supplemented as indicated) to each of the culture tubes. The preparation of the MS medium is given in Chapter 4. Plug the tubes with nonabsorbent cotton and cap with aluminum foil. The entire assembly is sterilized by autoclave and permitted to cool.

2. Apical shoots 1.0 cm in length are removed from the plant and placed in the hypochlorite solution for approximately 15 min. All subsequent procedures are conducted aseptically.

3. After surface sterilization, rinse the shoots several times in sterile DDH_2O. Transfer the shoots to a Petri dish containing Whatman No. 50 filter paper for surgical removal of the shoot apex. Moisten the filter paper with a few cubic centimeters of sterile DDH_2O to prevent desiccation of the shoot tips.

4. A binocular dissection microscope is necessary for viewing the shoot apex, and a magnification of 50× is adequate. The terminal 0.5 mm of the tip is carefully excised with a scalpel fitted with a pointed tip blade (no. 11). The excision should yield an explant consisting of the apical dome plus a small number of leaf primordia. In addition, several shoot tips should be removed varying in length from 1 to 5 mm. Observations will be made on the optimal size of the explant for purposes of micropropagation.

5. Each shoot tip is carefully transferred with the blade of the scalpel, or some type of microscalpel, to the depression in the filter-paper bridge as shown in Fig. 10.2.

6. The culture tubes, replugged and capped with foil, are cultured in a plant growth chamber set for 16/8-hr photoperiods at 27°C and a relative humidity of about 70%. An intensity of 1,000 lux is sufficient because the explants should not be exposed to high intensities of light (Shabde-Moses and Murashige, 1979).

RESULTS

The time required for the excised shoot tips to initiate growth and begin micropropagation, i.e., with the outgrowth of axillary buds, may vary from a few weeks to several months depending on the species and the cultural conditions.

Potato is one of the fastest growing plants in micropropagation. Within 5 to 6 weeks a dense cluster of proliferating shoots should be evident (Fig. 10.1). After the shoots are well developed they are excised and transferred to a root-induction medium. The latter medium is the same mixture that was used in this experiment except for the omission of BAP. Root initiation normally takes 4 to 6 weeks.

Once an adequate root system has developed the young plantlets are ready to be transferred to nonsterile conditions. The plantlets are first placed in a sterile soil mixture and maintained under humid conditions by mist irrigation. This is preferable to sealing the pots with plastic, which tends to encourage the growth of fungi. Gradually the young plants are hardened off by reducing the mist irrigation, and eventually they are

transferred to a cool greenhouse. Finally, the plants regenerated from the shoot-apex cultures are planted in the field.

QUESTIONS FOR DISCUSSION

1. Briefly describe the difference between the apical meristem and the shoot apex. Why are shoot apices preferred for the propagation of plants?
2. What are some problems associated with the growth and development of cultured shoot apices excised from woody plants?

APPENDIX

When whole plants have been obtained from the shoot-apex cultures, compare the growth rate of the cultured plants with similar plants produced by conventional methods. A comparison can be made by measuring the height, leaf area, dry weight, and so on, of the plants grown under the two conditions. Are there any significant differences between the two methods of propagation?

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11

Somatic embryogenesis

The capacity of flowering plants to produce embryos is not restricted to the development of the fertilized egg; embryos ("embryoids") can be induced to form in cultured plant tissues. This phenomenon was first observed in suspension cultures of carrot (*Daucus carota*) by Steward and co-workers (1958) and in carrot callus grown on an agar medium by Reinert (1959). This is a general phenomenon in higher plants, and experimental somatic embryogenesis has been reported in tissues cultured from more than 30 plant families (Raghavan, 1976; Narayanaswamy, 1977; Ammirato, 1983; see Table 11.1).

Somatic embryoids may arise in vitro from three sources of cultured diploid cells: (1) vegetative cells of mature plants, (2) reproductive tissues other than the zygote, and (3) hypocotyls and cotyledons of embryos and young plantlets without any intervening callus development (Kohlenbach, 1978). Precisely how these adventive embryoids arise from these tissues has been the subject of numerous studies. According to Sharp and his colleagues (1980), somatic embryogenesis may be initiated in two different ways. In some cultures embryogenesis occurs directly in the absence of any callus production from "preembryonic determined cells" that are programmed for embryonic differentiation. The second type of development requires some prior callus proliferation, and embryos originate from "induced embryogenic cells" within the callus (Sharp et al., 1980). Carrot cells are an example of the latter case. Although individual carrot cells are totipotent and carry all the genetic templates necessary for the development of the whole plant, isolated single cells do not generally become transformed into embryos by repeated divisions (McWilliam, Smith, and Street, 1974). Embryoids are initiated in callus from superficial clumps of cells associated with highly vacuolated cells that do not take part in embryogenesis. Observations have been made on the ultrastructure of embryogenic clumps of cells in callus derived from carrot (McWilliam et al., 1974; Street and Withers,

1974) and *Ranunculus* (Thomas, Konar, and Street, 1972). The embryoid-forming cells are characterized by dense cytoplasmic contents, large starch grains, and a relatively large nucleus with a darkly stained nucleolus. Staining reagents indicated that these embryogenic cells have high concentrations of protein and RNA. These cells also exhibited high dehydrogenase activity with tetrazolium staining (McWilliam et al., 1974; Street and Withers, 1974). Each developing embryoid passes through the sequential stages of embryo formation (i.e., globular, heart shape, and torpedo shape) (Fig. 11.1). Two critical events are involved in the early programming of this process: (1) the induction of cytodifferentiation of the proembryoid cells, and (2) the unfolding of the developmental sequence by these proembryoid cells (Kohlenbach, 1978). Although a given culture may differentiate these embryogenic cells, their further development may be blocked by an imbalance of chemicals in the culture medium. Abnormalities known as embryonal budding and embryogenic clump formation may occur, if relatively high levels of auxin are present in the medium after the embryogenic cells have been differentiated (Kohlenbach, 1978). In other words, two distinctly different types of media may be required, one medium for the initiation of the embryonic cells

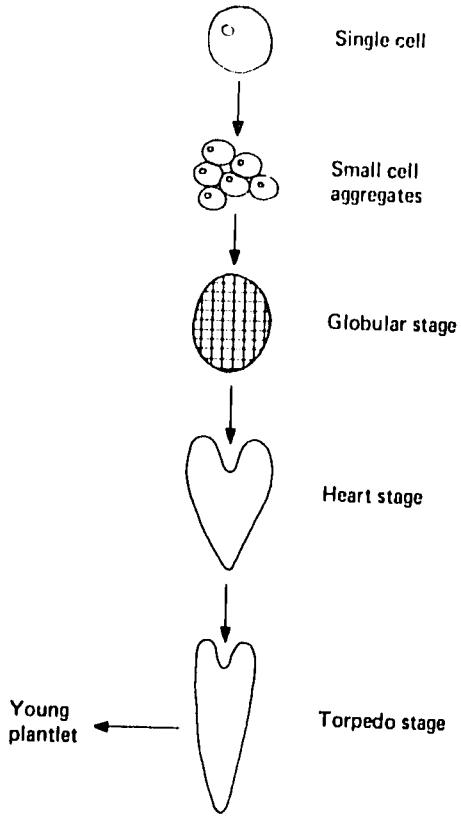
Table 11.1. *Examples of plants in which somatic embryogenesis has been induced under in vitro conditions*

Plant	Reference
Bromus inermis Leyss. var. Manchar	Gamborg et al., 1970
	Constabel et al., 1971
Brassica oleracea var. Borytis	Pareek and Chandra, 1978
Atropa belladonna L.	Konar et al., 1972
Carica papaya	Litz and Conover, 1983
Manihot esculentum	Stamp and Henshaw, 1982
Coffea arabica L.	Sondahl and Sharp, 1977
Pinus ponderosa Dougl.	Moore, 1976
Solanum melongena L.	Yamada et al., 1967
Daucus carota L.	Steward et al., 1958
	Reinert, 1959
	Halperin and Wetherell, 1964
	Homès, 1967
Citrus sinesis var. "Shamouti" orange	Kochba and Button, 1974
Hordeum vulgare	Bayliss and Dunn, 1979
Ranunculus sceleratus L.	Nataraja and Konar, 1970
Saccharum officinarum	Jane Ho and Vasil, 1983
Nicotiana tabacum var. Samsun	Lörz et al., 1977

and another for the subsequent development of these cells into embryoids. The first (induction) medium must contain auxin. The second medium generally consists of a mixture either lacking auxin, with a lower concentration of the same auxin, or with reduced levels of a different auxin. With some plants, however, both embryo initiation and subsequent maturation occurs on the first medium, and a second medium is employed for plantlet development (Ammirato, 1983).

The most important chemical factors involved in the induction medium are auxin and reduced nitrogen. Substantial amounts of reduced nitrogen are required in both the first and second media (Ammirato, 1983). In wild carrot cultures the addition of 10 mM NH_4Cl to an embryogenic medium already containing KNO_3 (12–40 mM) produced near-optimal numbers of embryoids. Glutamine, glutamic acid, urea, and alanine, respectively, were found to partially replace NH_4Cl as a supplement to

Fig. 11.1. Stages of somatic embryogenesis. Following repeated cell divisions, cell aggregates progressively develop and pass through globular, heart, and torpedo stages before ultimately forming plantlets.



KNO_3 (Wetherell and Dougall, 1976). These various nitrogen sources are not specific for the induction of embryogenesis, although at low concentrations organic forms are much more effective than inorganic nitrogen compounds.

The role of cytokinins in embryogenesis is somewhat obscure because of conflicting results. Although zeatin (0.1 μM) stimulates embryogenesis in carrot cell suspensions during the auxin-free subculture (secondary culture), the process is inhibited by the addition of either kinetin or benzylaminopurine to the medium (Fujimura and Komamine, 1975). The inhibitory effect of exogenous cytokinins may result from the increase in endogenous cytokinins in the developing embryoids (Al-Abta and Collin, 1979). Further information on cytokinins can be found in the review by Ammirato (1983).

Supplementing the medium with activated charcoal has facilitated embryogenesis in several cultures. The induction of embryogenesis was successful in *Daucus carota* cultures containing charcoal when auxin depletion failed to produce the desired results (Fridborg and Eriksson, 1975; Drew, 1979). Charcoal was a requirement for embryogenesis in English ivy (*Hedera helix*) cultures (Banks, 1979). Evidence indicates that charcoal may adsorb a wide variety of inhibitory substances as well as growth promoters (Ammirato, 1983). See Chapters 4 and 14 for additional comments on charcoal.

In general, embryogenesis occurs most readily in short-term cultures, and this ability decreases with increasing duration of culture (Reinert, Bajaj, and Zbell, 1977). There are exceptions, however, and embryogenesis has been reported in some cultures maintained over a period of years. Embryoid formation begins in carrot cultures about 4 to 6 weeks after isolation of the tissues, and an optimum embryogenic potential is reached after about 15 weeks (Reinert, Backs-Hüsemann, and Zerman, 1971). After the embryogenic potential has apparently been lost following 36 weeks in vitro, the carrot cultures can once again be induced to produce embryoids by transfer to an appropriate medium (Reinert et al., 1971). This temporary loss of embryogenic potential presumably results from the lack of biosynthesis of certain "embryogenic substances" by the cultured cells. In addition, changes in ploidy of the cultured cells may lead to a loss of morphogenetic potential (Smith and Street, 1974).

A technique has been developed for the physical separation of the globular, heart, and torpedo stages of embryogenesis by using glass beads to screen the cultures (Warren and Fowler, 1977). This procedure should prove useful for further biochemical studies of the developmental process.

Some progress has been made in inducing synchronization of somatic embryogenesis. A high degree of synchronization of embryogenesis was achieved in a carrot suspension culture by (1) sieving the initial cell populations, (2) employing density gradient centrifugation in Ficoll solutions, and (3) using repeated low-speed centrifugation for 5-sec periods. The resulting cell clusters, cultured in an auxin-free medium containing zeatin, gave a greater than 90% frequency of embryoid formation (Fujimura and Komamine, 1979).

Although the regeneration of whole plants by embryogenesis has been relatively rare in the Gramineae, somatic embyros have been formed directly from leaf mesophyll cells of orchard grass (*Dactylis glomerata* L.) without an intervening callus. Explants, prepared from the basal portions of two innermost leaves, were cultured on a Schenk and Hildebrandt medium containing 30 μM 3,6-dichloro-O-anisic acid (dicamba) and 0.8% (w/v) agar. Plantlet formation occurred after subculturing the embryos on the same medium lacking dicamba (Conger et al., 1983).

Endogenous polyamines appear to be required for the induction of embryogenesis in cultures of wild carrot (Feirer, Mignon, and Litvay, 1984). Embryogenic cultures of *Daucus carota* treated with a specific inhibitor of arginine decarboxylase showed a sharp reduction in embyro production compared to untreated controls. The cultures containing the inhibitor also had relatively low levels of the polyamines putrescine and spermidine. Supplementing the culture medium with either putrescine, spermidine, or spermine restored embryogenesis to the inhibitor-blocked cultures (Feirer et al., 1984).

LIST OF MATERIALS

Sterilization mode: C, chemical; O, oven; A, autoclave

suspension culture of carrot (*Daucus carota*) cells growing in an MS medium supplemented with 2,4-D (1.0 mg/l) and sucrose (3% w/v) (see Procedure)

A 250-cm^3 Erlenmeyer flask containing 200 cm^3 of culture medium (A) consisting of MS salts, zeatin (0.2 mg/l), and 2,4-D (0.1 mg/l)

A 250-cm^3 Erlenmeyer flask containing 200 cm^3 of culture medium (B) consisting of MS salts and zeatin (0.2 mg/l)

A 125-cm^3 Erlenmeyer flask containing 100 cm^3 DDH_2O

C scalpel

O forceps

9-cm disposable Petri dishes, plastic, commercially sterilized (10)

black card with 1-cm grid lines
A 2-cm^3 pipettes (six)
Parafilm sealing tape (one roll)
microscope slides; cover slips
light microscope (100 × magnification)
dissecting microscope (optional)
(Subsequent plantlet development requires filter-paper bridge apparatus and small pots containing sterile soil mixture.)

PROCEDURE

The preparation for this experiment starts with an actively proliferating callus initiated from the taproot of carrot as described in the procedure section of Chapter 5. According to Reinert and co-workers (1971), carrot callus does not develop the potential for embryogenesis until the culture has been growing for a minimum of 4–6 weeks after explant isolation. Healthy fragments of the callus are transferred to a liquid culture medium (see procedure section of chap. 9), and a carrot cell suspension is initiated. This liquid medium consists of basal MS salts supplemented with 2,4-D (1.0 mg/l) and sucrose (3% w/v).

Embryoids are initiated in the present experiment in the following manner:

1. Ten Petri dishes are prepared as follows: (a) five plates contain MS salts, zeatin (0.2 mg/l), 2,4-D (0.1 mg/l), sucrose (2% w/v), and agar (1% w/v); and (b) five plates contain MS salts, zeatin (0.2 mg/l), sucrose (2% w/v), and agar (1% w/v). The latter plates do not contain a source of exogenous auxin.

2. Aliquots (2 cm^3) of carrot suspension culture are added by pipette to the surface of the medium in the Petri dishes. The dishes are sealed with Parafilm and incubated at 25°C in the dark for 2–3 weeks.

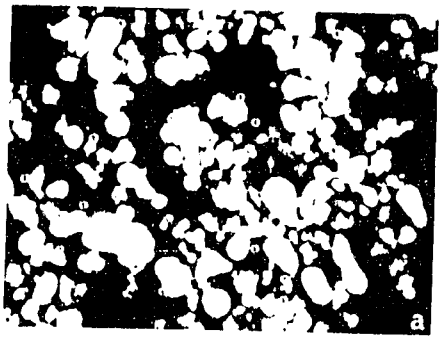
3. The test for embryogenic potential is based on a visual count of the embryoids. The "callus" from the agar surface is gently dispersed in DDH_2O, and the number of embryoids present is determined by placing the Petri dish over a black card marked with 1-cm^2 grid lines.

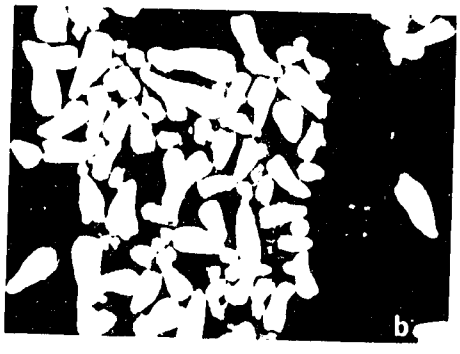
4. Small aliquots of this dispersed sample can be placed on a microscope slide and examined with a compound or dissecting microscope for the various stages of somatic embryogenesis.

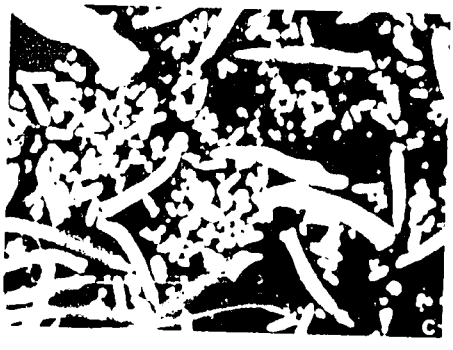
RESULTS

In the Petri dish containing the auxin medium the carrot cells develop into a callus and grow into small compact clumps. Embryoids, however,

Fig. 11.2. Stages of development of carrot (*Daucus carota*) embryoids. (a) Young globular stage. (b) Heart stage. (c) Torpedo stage. (d) Carrot plantlet growing on filter-paper bridge. (e) Mature carrot plant derived from cultured embryoid. (Courtesy of L. A. Withers.)







are not formed in these dishes. The carrot cells grown on the auxin-free medium produce large numbers of embryoids (Fig. 11.2). The embryoids are not formed in a synchronous manner. When an inoculum of this material is examined under the microscope, a wide range of developmental stages similar to those shown in Fig. 11.2a–c can be seen.

After the carrot cultures have reached the late torpedo stage of development, they can be transferred to filter-paper bridges (Fig. 11.2d). The use of the filter-paper bridge is described in the procedure of Chapter 10 (see Fig. 10.2). To the culture tubes add 3 cm^3 of liquid medium composed of MS salts, kinetin (0.2 mg/l), and sucrose (2% w/v). The plantlets that are formed can be potted in sterile soil and grown to maturity (Fig. 11.2e).

The plantlets must be maintained under a high relative humidity to prevent excessive water loss from the plantlets (see chap. 10).

QUESTIONS FOR DISCUSSION

1. What difficulties may be encountered with clonal propagation of plants by means of somatic embryogenesis?
2. What is the meaning of the term totipotency?
3. What is the genetic significance of totipotency?
4. What are the sequential stage of somatic embryogenesis?

APPENDIX

The following additional experiments may be of interest to some students.

1. Try a range of nitrogen : auxin ratios, and attempt to determine which combination yields the highest number of embryoids. Is there any difference between the use of an organic and inorganic nitrogen source?

2. The initial carrot cell suspension culture, which is to be plated on the embryogenic agar medium, is divided into three fractions by filtration: (a) an unfiltered fraction (mixed-cell suspension); (b) 75–200-μm fraction (single cells and small-cell aggregates); and (c) 75-μm fraction (single cells). Aliquots (2 cm^3) of each of these three fractions are plated as previously described in the procedure. After 3 weeks of growth, count the total number of embryoids produced by each of the fractions. Which fraction yields the greatest number of embryoids?

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12

Isolation, purification, and culture of protoplasts

Isolated protoplasts have been described as "naked" plant cells because the cell wall has been experimentally removed by either a mechanical or an enzymatic process. The isolated protoplast is unusual because the outer plasma membrane is fully exposed and is the only barrier between the external environment and the interior of the living cell (Cocking, 1972; Evans and Cocking, 1977). Despite technical difficulties that have limited their potential use in some investigations, protoplasts are currently utilized in several areas of study:

1. Two or more protoplasts can be induced to fuse and the fusion product carefully nurtured to produce a hybrid plant. Although this phenomenon has been observed repeatedly, fusion has not been achieved with the isolated protoplasts of some species. In some cases, hybrids that cannot be produced by conventional plant genetics because of sexual or physical incompatibility can be formed by somatic cell fusion (Power and Cocking, 1971; Cocking, 1977a; Dudits et al., 1979). The regeneration of *Atropa belladonna* plants from single isolated protoplasts is shown in Fig. 12.1.

2. After removal of the cell wall, the isolated protoplast is capable of ingesting "foreign" material into the cytoplasm by a process similar to endocytosis as described for certain animal cells and protozoans. Experiments are in progress on the introduction of nuclei, chloroplasts, mitochondria, DNA, plasmids, bacteria, viruses, and polystyrene beads into protoplasts (Davey and Cocking, 1972; Carlson, 1973; Cocking, 1977b; Cress, 1982; Lurquin and Sheehy, 1982; Dodds and Bengochea, 1983).

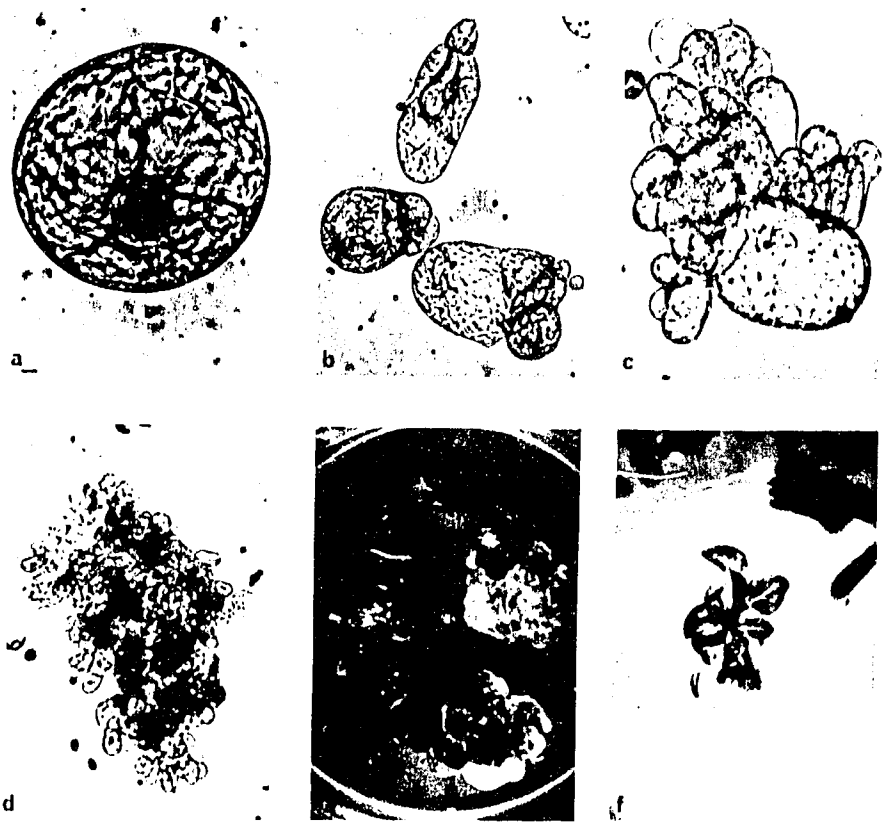
3. The cultured protoplast rapidly regnerates a new cell wall, and this developmental process offers a novel system for the study of wall biosynthesis and deposition (Willison and Cocking, 1972; Grout, Willison, and Cocking, 1973).

4. Populations of protoplasts can be studied as a single cellular system;

that is, their manipulation is similar to that of microorganisms (Evans and Cocking, 1977). Microbiological methods have been developed for the selection of mutant cell lines and the cloning of cell populations.

Most of the early research was conducted with the spongy and palisade mesophyll tissue obtained from mature leaves of *Nicotiana* and *Petunia*. With the improvement of techniques it has become possible to isolate protoplasts from a wide range of plant genera and tissues, for example, anthers of *Pelargonium* (Abo El-Nil and Hildebrandt, 1971), callus of *Gossypium hirsutum* (Bhojwani, Cocking, and Power, 1977), Crassulacean-acid-metabolism plants (Dodds, 1980), C_4 and C_3 plants (Kanai and Edwards, 1973), and *Solanum tuberosum* (Upadhya, 1975).

Fig. 12.1. Sequence of development of a plantlet of *Atropa belladonna* from a single isolated protoplast. (a) Single isolated protoplast. (b) Cell wall regeneration and initiation of cell division. (c,d) Development of cell aggregates. (e) Appearance of embryoids on surface of callus. (f) Formation of plantlet on agar medium. (Courtesy of H. Lörz.)



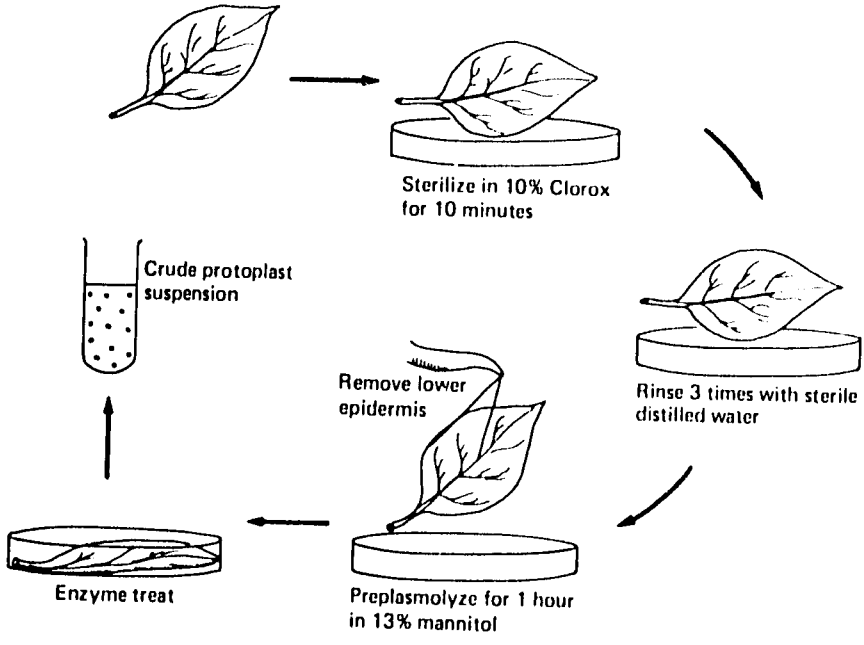
One must remember that a chief function of the cell wall is to exert a wall pressure on the enclosed protoplast and thus prevent excessive water uptake leading to bursting of the cell. Before the cell wall is removed, the cell must be bathed in an isotonic plasmolyticum, which is carefully regulated in relation to the osmotic potential of the cell. In general, mannitol or sorbitol (13% w/v) has given satisfactory results. A technique involving the use of a relatively low osmotic potential with sucrose (0.2 M) and polyvinylpyrrolidone (2% w/v) has been reported (Shepard and Totten, 1975). In developing an original technique it may be advantageous to test a range of mannitol concentrations varying from 8 to 15% (w/v) (Evans and Cocking, 1977). The latter authors have pointed out that preparations bathed in a plasmolyticum of too low a concentration may lead to multinucleate protoplasts owing to the spontaneous fusion of two or more protoplasts during the isolation procedure.

As mentioned previously, protoplasts can be released from the cell wall by either a mechanical or an enzymatic process. The mechanical approach involves cutting a plasmolyzed tissue in which the protoplasts have shrunk and pulled away from the cell wall. Subsequent deplasmolysis results in expansion and release of the protoplasts from the cut ends of the cells. In practice this technique is difficult and the yield of viable protoplasts is meager. One advantage, however, is that the complex and often deleterious effects of the wall-degrading enzymes on the metabolism of the protoplasts are eliminated. Since the early 1960s nearly all of the protoplast-isolation work has been performed with enzymatic procedures. By using enzymes one obtains a high yield ($2–5 \times 10^6$ protoplasts/g leaf tissue) of uniform protoplasts after removal of cellular debris (Evans and Cocking, 1977). The basic technique consists of (1) surface sterilization of leaf samples, (2) rinsing in a suitable osmoticum, (3) peeling off the lower epidermis or slicing the tissue to facilitate enzyme penetration, (4) sequential or mixed-enzyme treatment, (5) purification of the isolated protoplasts by removal of enzymes and cellular debris, and, finally, (6) transfer of the protoplasts to a suitable medium with the appropriate cultural conditions (Figs. 12.2 and 12.3).

The plant cell wall consists of a complex mixture of cellulose, hemicellulose, pectin, and lesser amounts of protein and lipid. Because of the chemical bonding of these diverse constituents, a mixture of enzymes would appear necessary to effectively degrade the system. Cellulose is a polymer consisting of subunits of D-glucose. Xylans form the bulk of the hemicellulose fraction in angiosperms (Northcote, 1972). These polymers consist, however, of several monosaccharides in addition to xylose.

Pectins are polysaccharides containing the sugars galactose, arabinose, and the galactose derivative galacturonic acid (Northcote, 1974). Protoplast isolation is achieved by using cellulase in combination with pectinase and hemicellulase. Most commercial preparations of these enzymes are isolated from microorganisms, and they often exhibit a variety of enzymatic activities. They may contain ribonucleases, proteases, and several other toxic enzymes. Because of the presence of these deleterious enzymes, as well as other harmful substances, several purification procedures have been developed. Although these techniques are beyond the scope of the present experiment, interested students can find detailed instructions in Patnaik, Wilson, and Cocking (1981), Constabel (1982), and Evans and Bravo (1983). There have been two approaches to the use of wall-degrading enzymes. In the mixed-enzyme method both pec-

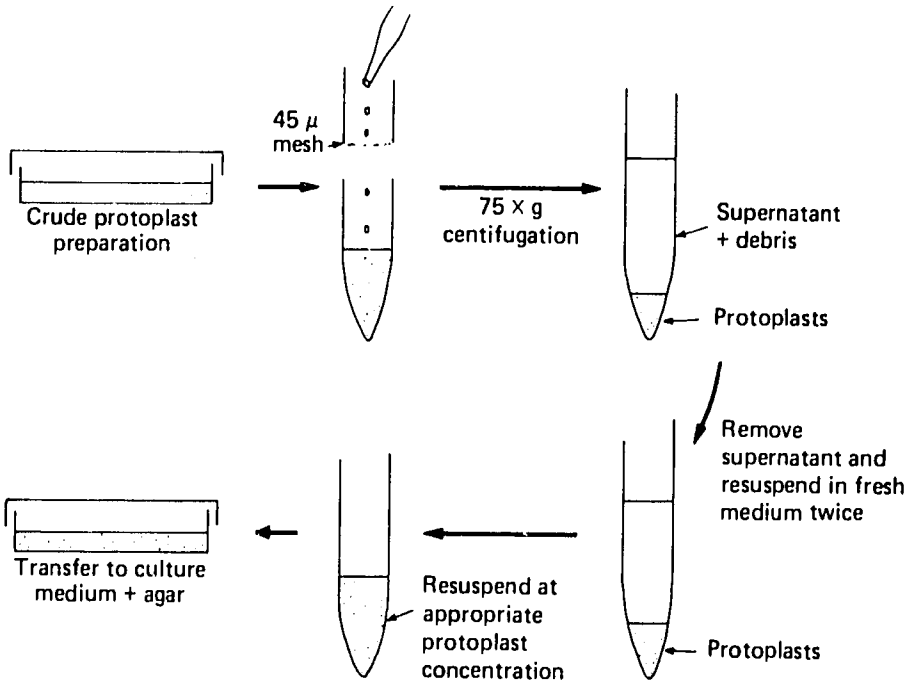
Fig. 12.2. Basic technique for the isolation of protoplasts from an excised leaf. The leaf is surface sterilized, rinsed repeatedly in sterile distilled water, and the cells are plasmolyzed in a solution of mannitol. The lower epidermal layer is stripped from the leaf to enhance enzyme penetration into the mesophyll tissue. Following treatment with one or more wall-degrading enzymes, a crude suspension of mesophyll protoplasts is obtained.



tinase and cellulase-hemicellulase are applied simultaneously, whereas the sequential method involves treatment of the leaf material with pectinase to loosen the cells, followed by a cellulase-hemicellulase digestion. Although both methods have certain advantages and disadvantages (Evans and Cocking, 1977; Bajaj, 1977), the mixed-enzyme technique will be used in the present experiment.

Each cellular system chosen for protoplast isolation presents its own unique problems in terms of isolation and culture procedures. Additional information on the selection of wall-degrading enzymes, as well as other variations in procedure, can be found in the Appendix. In this chapter

Fig. 12.3. Purification procedure for isolated protoplasts. The crude protoplast suspension is filtered through a nylon mesh 45-μm pore size), and the filtrate is centrifuged for 5 min at 75 × g. The supernatant, carefully removed by Pasteur pipette, is discarded. The protoplasts, resuspended in 10 cm^3 of fresh culture medium, are again centrifuged. Once again the supernatant is removed. The centrifugation and resuspension process is done three times. Before transfer of the protoplasts to a culture medium, the preparation is examined for protoplast density and viability (not shown).



the standard procedure for obtaining isolated protoplasts from mature leaf mesophyll tissue will be described. This technique can be employed with a reasonable degree of success on other tissues. In the following experiment the student will isolate, purify, and culture mesophyll protoplasts. An attempt will be made to regenerate plantlets from the protoplast-seeded callus.

LIST OF MATERIALS

Sterilization mode: C, chemical; O, oven; A, autoclave; U, ultrafiltration

C mature leaves from suitable plant (see Appendix)
paring knife
250 cm^3 aqueous solution (2.5% v/v) commercial bleach containing 2 cm^3 of detergent or wetting agent (1% v/v)
C scalpel
O forceps
U 10 cm^3 enzyme solution consisting of Macerozyme R-10 (0.5% w/v) plus cellulase Onozuka R-10 (2.0% w/v) dissolved in mannitol (13% w/v), pH 5.4
A 45-μm-pore-size nylon mesh
A Millipore filter holder or similar ultrafilter equipped with a membrane filter, 0.45-μm pore size
A 125-cm^3 Erlenmeyer flasks, each containing 100 cm^3 DDH_2O (six)
A 250-cm^3 Erlenmeyer flask containing 200 cm^3 MS medium plus mannitol (13% w/v)
A 250-cm^3 Erlenmeyer flask containing 200 cm^3 MS-mannitol (13% w/v) plus agar (1.5% w/v), 40°C
O 2-cm^3 pipettes
O 10-cm^3 pipettes with wide bore
O Pasteur pipettes with wide bore
O centrifuge tubes capped with aluminum foil, 6 × 16 mm
O white tile
Fuchs-Rosenthal hemocytometer, 0.2 mm in depth
ethanol (80% v/v) dip
ethanol (70% v/v) in plastic squeeze bottle
methanol lamp
bench-top centrifuge, swinging-bucket type
Evans blue dye reagent
fluorescein diacetate
fluorescence microscopy

Parafilm (one roll)
heavy-duty aluminum foil (one roll)

PROCEDURE

1. Mature healthy leaves are removed from the plants and rinsed briefly in tap water. The interior of the transfer chamber is wiped with a tissue soaked in ethanol (70% v/v), and all subsequent procedures are conducted under aseptic conditions. The leaves will be treated as shown in Fig. 12.2. Immerse the leaves in the hypochlorite–detergent solution for 10 min. As mentioned in the previous experiments, the UV lamp in the chamber should not be turned on during the hypochlorite sterilization. Rinse the leaves three times in order to remove all traces of the hypochlorite solution. Although the leaves may be rinsed in sterile DDH_2O, it is preferable to rinse them in a culture medium adjusted to the osmolality and pH of the enzyme solution to be used.

2. The waxy cuticle covering the leaves restricts access of the enzyme solution to the mesophyll cells. While the leaves are in the final rinse, the lower epidermis is peeled from the leaves with pointed forceps. The leaves are easier to peel if held on a sterile white tile. The point of the forceps is inserted at the junction of the main vein or midrib, and the epidermal layer is stripped toward the edge of the lamina. If this approach is unsuccessful, the leaves should be allowed to wilt before another attempt is made. If further attempts to peel the leaf fail, then the lower epidermis is scored several times with a scalpel to aid the penetration of the enzymes. Cut the leaves into small sections, and transfer approximately 1 g of peeled leaf strips to a Petri dish (100 × 15 mm) containing 10 cm^3 of sterile enzyme solution. The enzyme solution is sterilized by membrane filtration (0.45 μm). Although the types and concentrations of enzymes that may be employed will be discussed later, it is suggested for the initial experiment that the student use Macerozyme R-10 (0.5% w/v) plus cellulase Onozuka R-10 (2.0% w/v) dissolved in mannitol (13% w/v) at pH 5.4 (Power and Cocking, 1971). Seal the Petri dishes with Parafilm and wrap them with aluminum foil. Usually the leaf material is incubated in the enzyme solution overnight (12–18 hr, 25°C), although the mesophyll cells should be in contact with the enzymes for as short a time as possible. The leaf strips are then teased gently with forceps to release the protoplasts.

3. The protoplasts are purified by a combination of filtration, centrifugation, and washing (Fig. 12.3). First, the enzyme solution containing

the protoplasts is filtered through a nylon mesh to remove undigested tissue, cell clumps, and cell wall debris. Transfer the filtrate to a centrifuge tube and spin it at 75 × g for 5 min. The debris in the supernatant is carefully removed with a Pasteur pipette, the protoplasts having formed a pellet at the base of the tube. The protoplasts are resuspended in 10 cm^3 culture medium (complete MS plus mannitol, 13% w/v), and the process is repeated three times. The resuspension of the protoplasts must be carried out with considerable care with a wide-bore pipette (10 cm^3) in order to avoid injury. After the protoplasts have been examined for density and viability, they are ready for culture.

Determination of protoplast density and viability. Before the isolated protoplasts can be placed into culture it is necessary to examine them for viability with fluorescein diacetate. This dye, which accumulates only inside the plasmalemma of viable protoplasts, can be detected with fluorescence microscopy (Larkin, 1976; Strange, Pippard, and Strobel, 1982). Another staining method employs Evans blue. Intact viable protoplasts, by virtue of their intact plasmalemmas, are capable of excluding this biological stain. Impermeability of the cell to this dye presumably indicates a living cell (Kanai and Edwards, 1973). In addition, cyclosis or protoplasmic streaming has been reported to be a measure of viability (Raj and Herr, 1970).

Protoplasts have a maximum as well as a minimum plating density for growth. The optimum plating efficiency for tobacco protoplasts is about 5×10^4 protoplasts/cm^3; the protoplasts fail to divide when plated at one tenth of this concentration (Evans and Cocking, 1977). Other protoplasts have similar ranges; for example, isolated protoplasts of *Petunia* have an optimum plating density of 2.5×10^4 (Power et al., 1976). The concentration of protoplasts in a given sample can be determined by the use of a Fuchs-Rosenthal modified hemocytometer with a field depth of 0.2 mm. Hemocytometers designed for counting red blood cells normally have a field depth of 0.1 mm, which is too shallow for protoplasts isolated from large plant cells. By use of this instrument, it is possible to adjust the concentration of protoplasts to the appropriate level. In the present experiment we will assume a value similar to that for tobacco protoplasts. Because the protoplast preparation will be diluted by an equal quantity of agar-containing medium, the sample should be adjusted to a concentration of 10^5 protoplasts/cm^3.

Culture of protoplasts. Protoplasts have been cultured in several ways, for example, in hanging-drop cultures (Gleba, 1978), in microculture cham-

bers (Vasil and Vasil, 1973), and in a soft agar (0.75% w/v) matrix (Nagata and Takebe, 1971). The agar-embedding technique is one of the better methods as it ensures support for the protoplasts and permits observation of their development. The suspension of isolated protoplasts is adjusted with the hemocytometer by the addition of the culture medium plus mannitol (13% w/v), to yield a concentration of 10^5 protoplasts/cm^3. Five cubic centimeters of this liquid suspension of protoplasts is added to a Petri dish (100 × 15 mm). To this dish is then added 5 cm^3 of the complete MS medium plus mannitol (13% w/v); this latter solution also contains warm agar (1.5% w/v) in the sol state at 40°C. Care must be taken that the temperature does not exceed 45°C (Bajaj, 1977). The two aliquots are mixed by swirling the dish, and the final result is 10 cm^3 of a medium containing 5×10^4 protoplasts/cm^3. The culture of embedded protoplasts is incubated at 25°C in the presence of a dim white light.

Regeneration of plants from the protoplasts. Once the protoplasts have regenerated a cell wall, they undergo cell division and form a callus. This callus can be subcultured to plates or flasks containing a freshly prepared medium. If the callus of some species is transferred to a medium lacking both mannitol and auxin, embryogenesis begins on the callus after about 3–4 weeks (Kameya and Uchimiya, 1972; Lörz, Potrykus, and Thomas, 1977; Lörz and Potrykus, 1979). These embryoids, dissected from the callus, are nurtured in the same manner as the embryoids produced by somatic embryogenesis (see chap. 11) or by anther culture (see chap. 14). With proper care and attention the embryoids will develop into seedlings and eventually grow into mature plants.

Preparation of the culture medium. Follow the procedure outlined in Chapter 4 for the preparation of 1 liter of MS medium described for callus initiation. Because this medium contains an auxin and a cytokinin, it will readily induce growth and development in the isolated protoplasts. Add a suitable quantity of mannitol to the MS medium to yield a final concentration of 13% (w/v) mannitol. The function of mannitol is to prevent the osmotic lysis of the isolated protoplasts. Some of the medium also requires the addition of agar (1.5% w/v) before sterilization.

RESULTS

If green mesophyll tissue has been used for the isolation of protoplasts, the individual protoplasts will be readily visible with the use of bright field optics. This will also be true for pigmented protoplasts, such as

those from petals or pigmented storage tubers (*Beta vulgaris; Daucus carota*). If colorless tissue has been used, the naked protoplasts will be visible with the aid of phase-contrast optics.

When first placed into culture, the isolated protoplasts are spherical because of the lack of a rigid cell wall (Fig. 12.1a). Once they are in culture on a suitable medium, a cell wall is quickly reformed. After 5–7 days some of the cells begin to undergo cell division (Fig. 12.1b). Repeated cell division gives rise to clumps of cells (Fig. 12.1c,d), which eventually produce callus masses visible to the naked eye. Once callus is sufficiently large to be manipulated, it may be subcultured to a medium lacking mannitol and auxin. The latter medium induces the formation of embryoids (Fig. 12.1e), which may be nurtured to maturity (Fig. 12.1f).

QUESTIONS FOR DISCUSSION

1. What would be the result of transferring the isolated protoplasts to distilled water?
2. What is the advantage of using mannitol in preference to sucrose as an osmoticum?
3. List some of the possible applications of isolated plant protoplasts to the field of agriculture.
4. What are some advantages of using a mechanical technique over enzymatic digestion in the isolation of protoplasts?
5. What kinds of "foreign" material have been introduced into isolated protoplasts?

APPENDIX

Selection of plant material. With protoplast isolation it is important that considerable thought be given to the selection of an appropriate plant. In addition, the age, position on the plant, and environmental conditions will influence the suitability of a given leaf for protoplast isolation (Constabel, 1982). A reasonable degree of success can be achieved by employing leaves from either *Nicotiana tabacum* L. var. Xanthi (tobacco), *Petunia* sp., or *Hyoscyamus niger* (henbane). It is inadvisable, however, to employ any systemic fungicides or insecticides during the growth of these plants. Although protoplasts can be isolated from callus and cell suspension cultures, the techniques are not clearly defined. In beginning experiments involving protoplast isolation, it is advisable to select a leaf mesophyll tissue, at least until the techniques are mastered.

Selection of wall-degrading enzymes. At the present time a wide range of enzyme preparations are available for the digestion of the cell wall. De-

pending on the plant source and cellular type, varying concentrations of cellulase, hemicellulase, and pectinase will be required. A list of commercial preparations indicating the major enzymatic activity of each is given in Table 12.1. Typically, an enzyme preparation is chosen from each category. Some examples of mixtures of enzyme preparations that have been formulated for different plant material are given in Table 12.2. Additional formulations for various plant tissues can be found in the review by Evans and Bravo (1983).

Table 12.1. *Some enzyme preparations exhibiting wall-degrading activity classified according to major function*

Cellulases	Hemicellulases	Pectinases
cellulase Onozuka (R-10, RS) Cellulysin Meicelase (CESB, CMB) Driselase	Rhozyme HP-150 hemicellulase (Sigma)	Macerase Macerozyme R-10 Pectolyase Y-23 pectinase (Sigma)

Note: Commercial suppliers are given at the end of the book. Additional information on enzymatic activities is found in Terms, Abbreviations, and Synonyms.

Table 12.2. *Examples of combinations of enzyme preparations used successfully for the preparation of protoplasts*

Plant material	Enzyme mixture
Hemerocallis (cell suspension)	Cellulysin (1.0%), Rhozyme (1.0%), Macerase (0.5%)[a]
Pisum sativum L. (leaf mesophyll)	Onozuka R-10 (2.0%), Driselase (2.0%), Rhozyme (2.0%), pectinase (1.0%)[b]
Solanum sp. (leaf mesophyll)	Onozuka R-10 (1.0%), Macerozyme R-10 (0.5%), Pectolyase Y-23 (0.013%)[c]
Medicago sativa (root; cotyledon)	Meicelase (4.0%), Rhozyme (2.0%), Macerozyme R-10 (0.03%)[d]

[a] Fitter and Krikorian (1983).
[b] Constabel (1982).
[c] O'Hara and Henshaw (1982).
[d] Lu et al. (1982).

Variability of cultural conditions. Results may differ depending on the commercial agar employed and the concentration used in the culture medium. For example, an agar concentration as low as 0.2% (w/v) has given good results (Binding, 1974). Protoplast division is sensitive to temperature, and some mesophyll protoplasts may respond better at incubation temperatures higher than 25°C. Another cultural variable is light. Although the present experiment was conducted with dim light, the plating efficiency may improve with higher light intensities after the first 48 hr of culture. On the other hand, some mesophyll protoplasts respond better in complete darkness (Evans and Cocking, 1977). Another interesting possibility is enrichment of the culture medium with various organic growth factors (e.g., coconut milk, carbohydrates, organic acids, and casamino acids) (Kao and Michayluk, 1975).

Role of divalent cations in membrane stability. During the initial period of isolation and culture, protoplasts appear to benefit from a higher level of divalent cations than is normally present in a typical tissue culture medium. The MS medium may be supplemented with $CaCl_2 \cdot H_2O$ (5–10 mM) alone or in some combination with $CaH_4(PO)_2 \cdot H_2O$ (Constabel, 1982). Mesophyll protoplasts isolated from *Petunia* grew better, according to Binding (1974), with the medium supplemented with a combination of $CaCl_2$ (5 mM) and $Mg(SO_4)$(4mM).

Observations on cell wall regeneration. Mesophyll protoplasts start to regenerate a new cell wall within a few hours following isolation, although it may take several days to complete wall biosynthesis. These initial events occurring on the surface of the plasmalemma can be observed microscopically by using Calcofluor White M2R, purified (see Commercial sources of supplies). This white dye binds to wall material and exhibits fluorescence on irradiation with blue light. The regenerating cells are incubated in 0.1% (w/v) Calcofluor dissolved in the appropriate osmoticum for 5 min (Evans and Cocking, 1977). After rinsing to remove excess dye, the protoplasts can be examined microscopically. Cellulose layers will fluoresce when irradiated with UV light at 366 nm (Constabel, 1982). A mercury vapor lamp with excitation filter BG12 and suppression filter K510 may be employed as a light source.

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13

Protoplast fusion and somatic hybridization

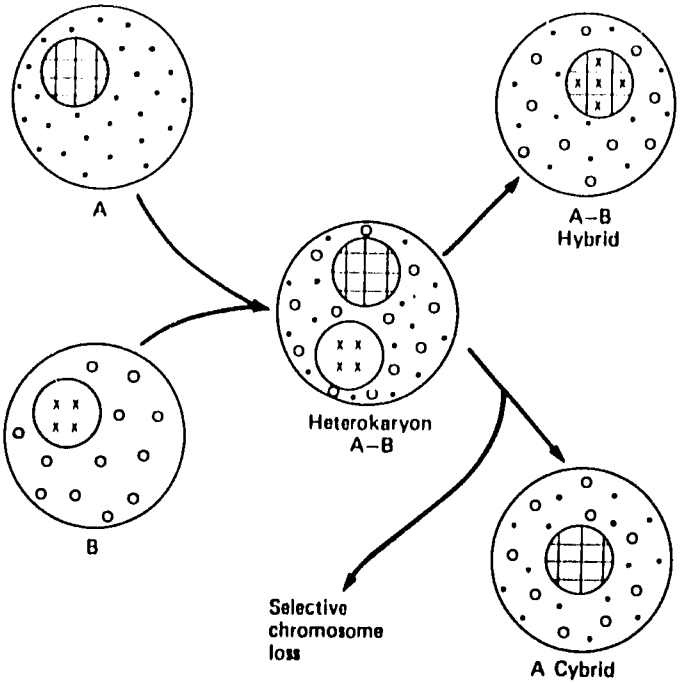
The preceding chapter describes the methods used for the isolation, purification, and culture of isolated protoplasts, and offers some insight into the way in which whole plants may be regenerated from single isolated protoplasts. The interest in protoplast fusion techniques is related to the prospect that wider crosses than are now possible by sexual means may be achieved with protoplast fusion. For example, some plants that show physical or chemical incompatibility in normal sexual crosses may be produced by the fusion of protoplasts obtained from two cultures of different species. It should be emphasized, however, that hybrid whole plants have been regenerated in a relatively small number of fusion systems, and there are no instances of the successful use of somatic hybridization in a plant-breeding program. In the early research work, protoplasts could be isolated from a small number of plants; the number of successful protoplast isolations increased following improvements in technique. Doubtless the number of successful fusion experiments will rapidly increase after the techniques have been perfected.

The fusion of plant protoplasts is not a particularly new phenomenon; Küster in 1909 described the process of random fusion in mechanically isolated protoplasts. When two or more isolated protoplasts are fused together, there is always a coalescence of the cytoplasms of the various protoplasts (Fig. 13.1). The nuclei of the fused protoplasts may fuse together, or they may remain separate. Cells containing nonidentical nuclei are referred to as heterokaryons or heterokaryocytes (Mastrangelo, 1979). The fusion of nuclei in a binucleate heterokaryon results in the formation of a true hybrid protoplast or synkaryocyte (Constabel, 1978). The fusion of two protoplasts from the same culture results in a homokaryon. Frequently genetic information is lost from one of the nuclei. If one nucleus completely disappears, the cytoplasms of the two parental protoplasts are still hybridized (Fig. 13.1), and the fusion product is known as a "cybrid" (cytoplasmic hybrid; heteroplast). Certain

genetic factors are carried in the cytoplasmic inheritance system instead of in the nuclear genes (e.g., male sterility in some plants). The formation of cybrids, therefore, has application in a plant-breeding program.

Spontaneous fusion of protoplasts may occur, or they may be induced to fuse in the presence of "fusigenic agents." During enzymatic digestion of the cell wall, the protoplasts of contiguous cells may fuse together through their adjoining plasmodesmata. The dissolution of the wall allows the plasmodesmatal strands interconnecting the cells to enlarge; the cytoplasm and organelles from two or more cells then flow together. These spontaneous fusions are always intraspecific (i.e., originate from the same plant tissue). This phenomenon rarely occurs because the negative charges on the surface of the protoplasts cause them to repel each other (Grout and Coutts, 1974; Nagata and Melchers, 1978). Although the fusigenic agent lowers the surface charge, which permits the protoplast membranes to come into proximity, the adhesion of the proto-

Fig. 13.1. Some fusion products resulting from protoplast culture. The fusion of protoplasts A and B results in a binucleate heterokaryon containing the cytoplasmic contents of the two original protoplasts. Fusion of the two nuclei results in a tetraploid hybrid cell or synkaryocyte. If one of the nuclei degenerates, a cybrid or heteroplast is produced.



plasts is insufficient to bring about fusion without molecular alterations in the bilayer structure of the plasma membranes (Ahkong et al., 1975a).

Several compounds have been shown to have a fusigenic effect on protoplasts. In 1970 Power and co-workers, University of Nottingham, showed that the addition of sodium nitrate to the culture medium induced fusion of root protoplasts from oat (*Avena sativum* L.) and maize (*Zea mays* L.). Fusion is also promoted by a combination of high pH (10.5), a high concentration of calcium ions (50 mM $CaCl_2 \cdot 2H_2O$), and high temperature (37°C) (Keller and Melchers, 1973; Melchers and Labib, 1974). At the present time polyethylene glycol (PEG) is the most widely used fusigenic agent (Constabel and Kao, 1974; Kao and Michayluk, 1974). The molecular weight and concentration of PEG, the density of protoplasts, the incubation temperature, and the presence of divalent cations are all factors that play a role in the fusion process (Wallin, Glimelius, and Eriksson, 1974). Polyethylene glycol has been used to induce the fusion of animal cells (Pontecorvo, 1975); therefore, it was not surprising that a heterokaryon was produced between an animal cell and a plant cell (Ahkong et al., 1975b). The latter heterokaryon involved the fusion of a hen erythrocyte and a yeast protoplast. The fusion of cultured amphibian cells with protoplasts of a higher plant has also been reported (Davey et al., 1978).

The experiment outlined in this chapter involves an attempt to induce fusion between isolated protoplasts from two different plant sources. The inductive treatment, which involves low-speed centrifugation of the mixed culture in the presence of polyethylene glycol (PEG), will result in a range of fusion products. The protoplast population will consist of unfused parental cells from the two tissues, homokaryons, heterokaryons, and multiple fusion products. If this mixture is plated on a culture medium, some of the various cell types will divide and develop callus. The next problem for the investigator is the recognition of callus formed by somatic hybrid and cybrid cells. The selection procedures are generally of two types: visual and biochemical selection methods.

Visual selection has been restricted to the fusion of colorless protoplasts with protoplasts containing chloroplasts. Protoplasts that demonstrate the completely integrated structural characteristics of both parental types are heterokaryons and potentially hybrid cells (Kao et al., 1974; Power and Cocking, 1977; Patnaik et al., 1982). An example of the fusion of plant protoplasts is shown in Fig. 13.2. It is possible to attach a fluorescent label to the outer membranes of two parent protoplast populations and then separate the fusion products from the pa-

Fig. 13.2. Fusion of colorless and chloroplast-containing protoplasts. (Courtesy Professor G. Melchers.)

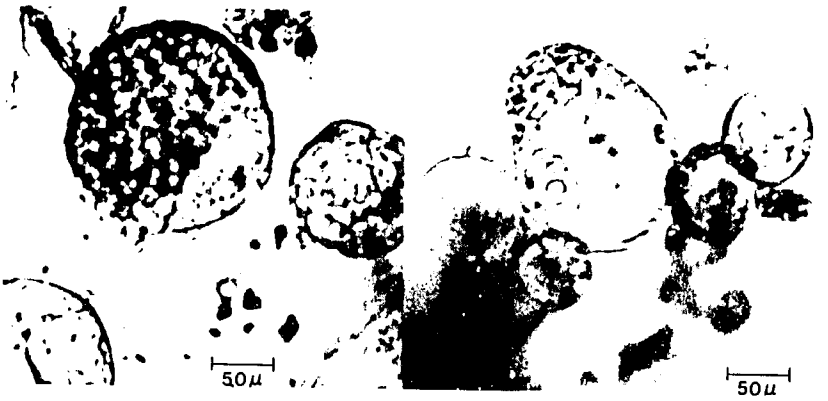
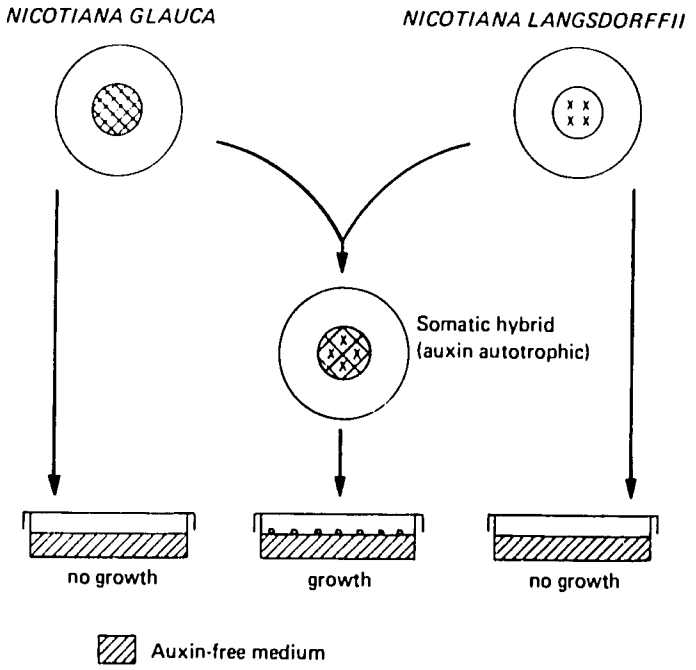


Fig. 13.3. Screening method for the detection of somatic hybrids that are auxin-autotropic in nutrition. The isolated protoplasts of both *Nicotiana glauca* and *N. Langsdorffii* are unable to grow in the absence of exogenous auxin. The fusion product of the two parental types is auxin-autotropic and grows on an auxin-free medium.



rental mixture by a method of fluorescent cell sorting (Redenbaugh et al., 1982).

Somatic hybrids can be selected by using a medium to encourage selective growth. A method was developed for the selection of hybrids resulting from the fusion of protoplasts of *Nicotiana glauca* and *N. Langsdorffii* (Carlson, Smith, and Dearing, 1972; Fig. 13.3). Neither of the parental protoplasts is capable of growth on a medium deprived of auxin. The protoplast fusion products of the two species are auxin autotropic, and they are capable of callus formation on an auxin-free medium. The callus can then be subcultured and induced to regenerate hybrid plants. The rationale for this procedure was later confirmed by Smith, Kao, and Combatti (1976).

Another method involves the use of biochemical mutants and a selection of somatic hybrids by a form of complementation. The antibiotic actinomycin D was used in the detection of fusion products of two species of *Petunia* (Power et al., 1976). Cultured cells of *P. hybrida* cannot grow in the presence of actinomycin D, whereas cells of *P. parodii* are capable of growth in the presence of this antibiotic. The cells of the latter species, however, are unable to regenerate *Petunia* plants from callus cultures. The only cells capable of growth in the presence of actinomycin D and capable of regeneration of whole plants are the fusion products of the two parental protoplast lines. Two mutant strains of *Nicotiana tabacum*, which have the characteristics of light sensitivity and chlorophyll deficiency, were used in a complementation selection procedure by Melchers and Labib (1974).

The techniques outlined in this chapter are probably among the most difficult that the student will encounter in this book. The fusion experiment should not be attempted until protoplasts have been successfully isolated and cultured (see chap. 12). The present experiment involves the induction of fusion of two protoplasts carrying distinct visual markers in the form of pigment color. The fusion products can be identified by microscopic examination of the cells. After this technique is mastered, the student can advance to more complex hybridization and selection methods.

LIST OF MATERIALS

Sterilization mode: C, chemical; O, oven; A, autoclave

isolated protoplasts of two types in culture medium (chap. 12). Using the methods of the previous chapter, isolated protoplasts from one of the following plants:

(a) leaves and tubers of *Beta vulgaris*
(b) leaves and taproot of *Daucus carota*
(c) leaves and petals of *Nicotiana tabacum*
(d) leaves and petals of *Petunia hybrida*

O 9-cm Petri dishes (10)
O forceps
O centrifuge tubes, 6 × 16 mm (capped with aluminum foil)
A 250-cm^3 Erlenmeyer flask with 200 cm^3 MS-mannitol (13% w/v) plus agar (2% w/v), 40°C (see procedure in chapt. 12)
A polyethylene glycol solution (20% w/v; molecular weight 3,000)
A Pasteur pipettes, wide bore
Fuchs-Rosenthal hemocytometer, 0.2 mm in depth
ethanol (80% v/v) dip
ethanol (70% v/v) in plastic squeeze bottle
methanol lamp
bench-top centrifuge, swinging-bucket type
Evans blue dye reagent
fluorescein diacetate
fluorescence microscopy

PROCEDURE

1. After selection of the appropriate plants, isolate two sets of protoplasts by employing the technique outlined in the previous chapter. This procedure should result in one tube containing green protoplasts of mature leaf material and a second tube of red protoplasts from petal, tuber, or taproot tissue. In other words, the markers for fusion are chloroplasts and vacuoles containing anthocyanin pigment.
2. The basic principle of fusion is shown in Fig. 13.1. Similar numbers of protoplasts (A) and (B) are mixed in a centrifuge tube containing PEG (20% w/v) as the fusigenic agent. The tube is centrifuged at 75–100 × g for 10 min. This relatively slight pressure forces the protoplasts into close contact and allows fusion to take place.
3. The pellet of fused and unfused protoplasts is carefully resuspended and assayed for viability and density, and the mixture is plated out as described in Chapter 12.

RESULTS

After the protoplast mixture has been plated and the agar has solidified, the plate may be viewed microscopically for the identification of the fusion products. Heterokaryons can be identified by the presence of

chloroplasts and an anthocyanin-containing vacuole. There are many different combinations of fusion products, and the student should attempt to identify as many as possible. If any of the fusion products initiates a callus, plant regeneration can be attempted as previously described in Chapter 6.

QUESTIONS FOR DISCUSSION

1. What chemical compounds have been employed as fusigenic agents?
2. What are the advantages of protoplast fusion over traditional methods of sexual hybridization?
3. What types of procedures can be used for the selection of hybrid cells?
4. What is a cybrid? How does this phenomenon occur, and does it have any significance in the breeding of plants?
5. List some interesting protoplast fusions that might result in unusual hybrids (e.g., potato and tomato).

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14

Anther and pollen cultures

The cells of haploid plants contain a single complete set of chromosomes, and these plants are useful in plant-breeding programs for the selection of desirable characteristics. The phenotype is the expression of single-copy genetic information, there being no masking of a trait through gene dominance. The purpose of anther and pollen culture is to produce haploid plants by the induction of embryogenesis from repeated divisions of monoploid spores, either microspores or immature pollen grains. The microspore represents the beginning of the male gametophyte generation. Mature microspores, especially following their release from tetrads, are referred to as pollen grains (Bhojwani and Bhatnagar, 1974). The chromosome complement of these haploids can be doubled by colchicine or by regeneration techniques to yield fertile homozygous diploids (Vasil and Nitsch, 1975). Although the number of successful pollen culture systems is still relatively small (Sunderland, 1971, 1977), this technique has resulted in several improved varieties of crop plants in China (Sunderland and Cocking, 1978; Sunderland, 1979).

Tulecke (1953) first observed that mature pollen grains of the gymnosperm *Ginkgo biloba* could be induced to form a haploid callus following culture on a suitable medium. Repeated divisions of cultured pollen grains of angiosperms were described much later by Guha and Maheshwari (1966), who made a remarkable discovery by accident. These investigators were conducting experiments with cultured pollen grains of *Datura innoxia* in order to determine the feasibility of this system for the study of factors regulating meiosis. The growth response of the pollen grains, enclosed in mature anthers, was of three types and reflected the nature of the medium. Although the pollen grains were unresponsive in the presence of IAA, callus was initiated on media containing either yeast extract or casein hydrolysate. Torpedo-shaped embryoids, which later developed into plantlets, were produced following culture of the anthers on media containing either kinetin or coconut

milk. Acetocarmine staining revealed that the newly formed plantlets contained only a single set of chromosomes (Guha and Maheshwari, 1966, 1967).

The particular stage of development of the anthers at the time of culture is the most important factor in achieving success in the formation of embryoids. In angiosperms with an indeterminate number of anthers in each flower bud, buds can be selected that will contain several anthers in various stages of pollen development. In species with a determinate number of anthers per flower, a series of buds must be examined in order to give all the stages of development. Two basic methods are used: (1) Excised anthers are cultured on an agar or liquid medium, and embryogenesis occurs within the anther; or (2) the pollen is removed from the anther, either by mechanical means or by natural dehiscence of the anther, and the isolated pollen is cultured on a liquid medium. It may take 3–8 weeks for haploid plantlets to emerge from the cultured anthers (Reinert and Bajaj, 1977).

Sunderland (1979) reported that in flowers of many plants anthers fall into one of three categories: premitotic, mitotic, or postmitotic. In the premitotic category the best response is obtained by using anthers in which the microspores have completed meiosis, but have not yet started the first pollen division (e.g., *Hyoscyamus*, *Hordeum vulgare*). Anthers of plants belonging to the mitotic group respond optimally about the time of the first pollen division (e.g., *Nicotiana tabacum*, *Datura innoxia*, *Paeonia*). The early bicellular stage of pollen development is best in the postmitotic plants (e.g., *Atropa belladonna*, *Nicotiana* species). In the case of *N. tabacum*, floral buds with the corolla barely visible beyond the calyx will probably contain anthers at the appropriate stage of development, although there may be slight differences between different cultivars (Kasperbauer and Wilson, 1979). This particular stage has been termed "stage-2" by Nitsch (1969) and "stage-4" by Sunderland and Dunwell (1977).

As discussed previously in Chapters 4 and 11, activated charcoal has a stimulatory effect on somatic embryogenesis as well as the initiation of embryos from haploid anther tissue. This charcoal effect has been demonstrated for anther cultures of tobacco (Anagnostakis, 1974; Bajaj, Reinert, and Heberle, 1977), rye (Wenzel, Hoffmann, and Thomas, 1977), potato (Sopory, Jacobsen, and Wenzel, 1978), and other plants. The removal of inhibitory substances from the agar is considered to be a factor, since a similar response was obtained by dialyzing agar against activated charcoal or by employing highly purified agar (Kohlenbach and

Wernicke, 1978). Another possibility is adsorption by charcoal of 5-(hydroxymethyl)-2-furfural, a degradation product of autoclaved sucrose (Weatherhead, Bordon, and Henshaw, 1978). A study of anther cultures of *Petunia* and *Nicotiana* was consistent with the hypothesis that charcoal removes both endogenous and exogenous growth regulators from the culture medium (Martineau, Hanson, and Ausubel, 1981). Although the precise role of activated charcoal in this developmental process remains unknown, the use of charcoal for the enhancement of haploid plantlet production should be encouraged (Bajaj, 1983).

Another factor in anther culture is the physiological status of the parent plant (e.g., photoperiod, light intensity, temperature, and mineral nutrition). Anthers should be taken from flowers produced during the beginning of the flowering period of the plant (Sunderland, 1971). Higher yields of embryos have been reported from donor plants grown under short days and high light intensities (Dunwell, 1976). For additional information see Sunderland and Dunwell (1977).

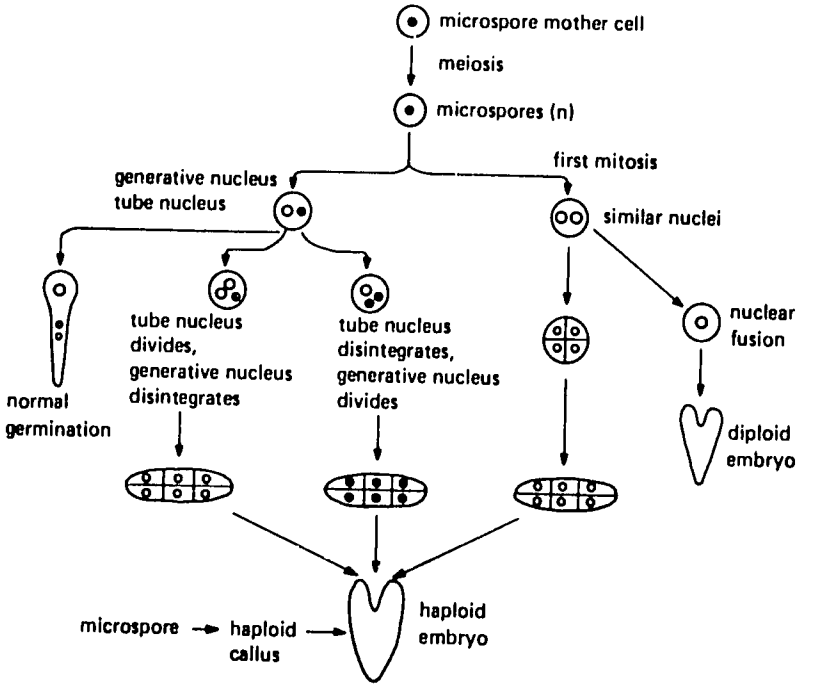
Various types of anther pretreatment have been found to improve embryo production in some plants. Low-temperature pretreatment of anthers for periods of 2–30 days at temperatures of 3–10°C may stimulate embryogenesis (Sunderland and Roberts, 1977). Other types of pretreatment include soaking the detached inflorescences in water for several days (Wilson, Mix, and Foroughi-Wehr, 1978) and centrifugation of the anthers at 3–5°C for approximately 30 min (Sangwan-Norreel, 1977).

The presence of anther tissue in the culture introduces several ill-defined factors that influence embryoid production. Raghavan (1978) suggested that a gradient of endogenous auxin within the anther may play a role in pollen grain development. Embryogenic pollen grains were observed to be confined to the periphery of the anther locule in close proximity to the tapetum, and possibly substances released from the tapetum initiate embryogenic divisions in pollen grains within cultured anther segments of *Hyoscyamus niger* (Raghavan, 1978). Pollen is also sensitive to toxic substances released following injury to anther wall tissue (Horner and Street, 1978).

It is important to determine the chromosome number of the newly formed plantlets because there may be considerable variation in ploidy levels, depending on the developmental events that led to embryoid formation. Diploid heterozygous plants may arise from anther tissue or from growth of the microspore mother cells and unreduced microspores. The further development of dyads and incomplete tetrads often produces

plants that are heterozygous at certain loci because of crossing-over prior to the first reduction division. Chromosome doubling and nuclear fusion can produce homozygotes with varying ploidy levels. Plantlets formed from the callus tissue arising from haploid microspores can exhibit mutations and chimeras (Thomas and Davey, 1975). It was found that the ploidy levels of 2,496 rice plants derived from pollen cultures were 35.3% haploid, 53.4% diploid, 5.2% polyploid, and 6.0% mixoploid (Hu Han et al., 1978). Some developmental pathways exhibited by microspores are shown in Fig. 14.1.

Fig. 14.1. Some possible developmental pathways of microspores under in vivo and in vitro conditions. Normal development (in vivo) results in the production of two sperm, a tube nucleus or cell, and pollen tube formation (far left). Several possibilities exist following the in vitro culture of isolated microspores or anthers. Either the tube or generative nucleus degenerates, and the surviving nucleus divides repeatedly and ultimately produces a haploid embryoid. The first mitosis of the microspore may produce two similar nuclei, and repeated division of these nuclei can produce a haploid embryoid. The similar nuclei, however, may fuse and produce a diploid embryoid (far right). Haploid callus of microspore origin can form embryoids de novo. (Diagram modified from Devreux, 1970.)



There are several techniques for doubling the chromosome number of the haploid plants, and two approaches can be taken: (1) regeneration by tissue culture methods, and (2) chemically induced doubling with colchicine. The ploidy level of the plant involved must first be confirmed with standard cytological procedures before additional experiments are undertaken (Darlington and LaCour, 1976; Collins. 1979). One method of chromosome doubling employs aged leaf tissue from haploid plants because older leaves have the potential to regenerate both haploid and diploid plants. The diploids result from chromosome endoreduplication, which frequently occurs in cultured plant tissues. A technique for inducing plantlet formation starting with leaf explants from haploids has been given by Kasperbauer and Wilson (1979). In addition, the chromosome number can be doubled by the application of colchicine to either the embryos or the haploid plants. A simple procedure is to immerse the anthers containing the newly formed plantlets in an aqueous solution of colchicine (0.5% w/v) for 24–48 hr (Burk, Gwynn, and Chaplin, 1972). Another approach is to apply a preparation of colchicine in lanolin paste (0.4% w/v) to the axillary buds of decapitated mature haploid plants (Tanaka and Nakata, 1969). An evaluation of the technique for chromosomes is given by Jensen (1974).

The potential for using haploid plants and homozygous lines in plant breeding programs has long been recognized. One important area of research concerns the development of homozygous lines for the production of hybrids in self-incompatible species (e.g., rye and rape) (Keller and Stringham, 1978). Microspore culture is important in mutagenic studies. Mutations are not masked in haploids because there cannot be a dominant gene. For these studies to be successful, however, large numbers of microspores must be induced to undergo embryogenesis and develop on nutrient media. The haploids must remain genetically stable, and it must be possible to regenerate diploid plants from the haploids. This has been achieved in the case of *Nicotiana tabacum* (Devreux and Laneri, 1974).

Three different techniques will be introduced in this chapter. In the first experiment the student will culture anthers of either henbane (*Hyoscyamus niger*) or tobacco (*Nicotiana tabacum*). Both of these plants are in the Solanaceae family. An attempt will be made in the second experiment to induce the formation of haploid plantlets via the culture of isolated pollen removed from excised anthers. Assuming success in the previous experiments, the student will then attempt to obtain chromosome doubling and the production of diploid plants from the haploids.

LIST OF MATERIALS

Sterilization mode: C, chemical; O, oven; A, autoclave

plants of *Hyoscyamus niger* or *Nicotiana tabacum* in the flower bud stage of development
C scalpel, equipped with narrow blade (Bard-Parker no. 11)
O forceps (two)
O dissecting needles prepared from soft glass rod
O 9-cm Petri dishes (10)
O culture tubes required for experiment 1 (10)
O 5-cm^3 pipettes
O Pasteur pipettes
A 125-cm^3 Erlenmeyer flasks, each containing 100-cm^3 DDH_2O (five)
A 100-cm^3 MS medium supplemented with sucrose (2% w/v) and agar (0.6–0.8% w/v) required for experiment 1
A 50-cm^3 liquid MS medium supplemented with sucrose (2% w/v) required for experiment 2
100 cm^3 aqueous solution (10% v/v) commercial bleach containing 2 cm^3 detergent or wetting agent (1% v/v)
ethanol (80% v/v) dip
ethanol (70% v/v) in plastic squeeze bottle
methanol lamp
dissection microscope
microscope slides; cover slips
acetocarmine stain
Fuchs-Rosenthal hemocytometer, 0.2 mm in depth
incubator or refrigeration unit for chilling buds (preset 7–8°C)
growth chambers equipped with fluorescent lighting (300 lux; 5,000 lux)
root-induction medium (required after 4–5 weeks)
colchicine (0.5% w/v)
Parafilm (one roll)

PROCEDURE

Experiment 1. Anther culture

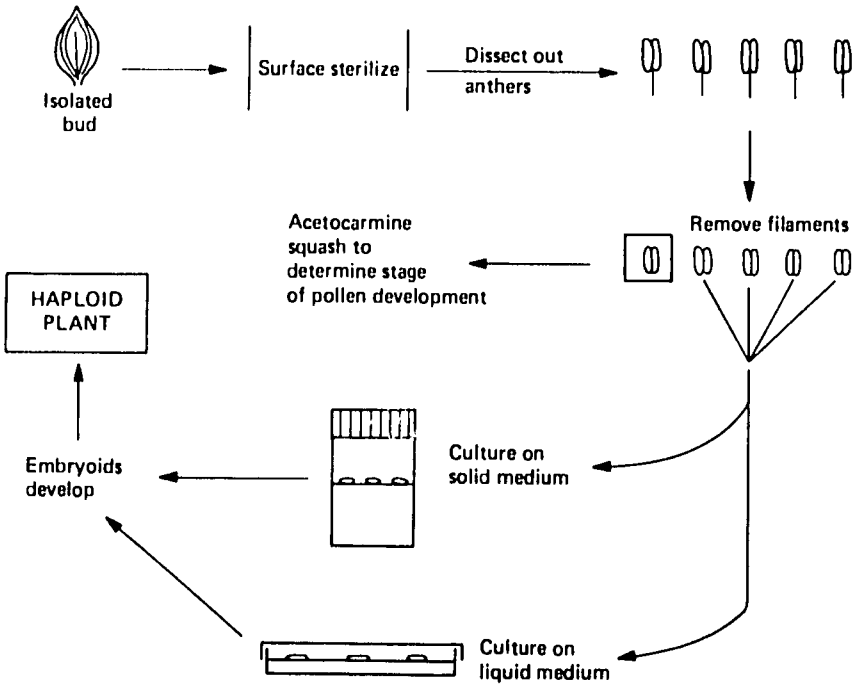
1. Plants selected for experimentation are cultivated until they reach the flower bud stage. In the case of tobacco plants the buds should have a corolla length of 21–23 mm, and at this stage the pollen will have completed the first mitosis (Sunderland and Roberts, 1977). The methods used for isolation and culture are shown in Fig. 14.2.

2. If tobacco is employed, best results will be obtained by chilling the buds approximately 12 days (7–8°C) prior to culture (Sunderland and Roberts, 1977). For surface sterilization the buds are transferred to a Petri dish containing hypochlorite solution plus a wetting agent (10 min).

3. Rinse the buds several times in sterile DDH_2O. Using forceps and a dissecting needle, carefully tease open the buds and remove the anthers. Great care is required, and a dissection microscope is necessary for this step. The dissected anthers from each bud are grouped together as they are removed.

4. One anther from each group is removed and squashed in aceto-carmine in order to determine the stage of pollen development. If the pollen in the squashed anther is in the corect stage of development, then the remaining anthers from that bud are placed into culture. In the case of *Hyoscyamus niger* the microspores should have completed meiosis but not yet initiated the first pollen division. The pollen of *Nicotiana tabacum*

Fig. 14.2. Basic procedure for the production of haploid plants from anther culture. Isolated buds are surface sterilized and the anthers aseptically removed. Individual anthers are screened by an acetocarmine staining method for the selection of the proper stage of pollen development. Subsequent culture results in embryoid and haploid plantlet formation.



should exhibit the first pollen division. The filaments must be removed prior to culture, or they will form callus at the cut ends.

5. Anthers can be cultured either on agar-solidified culture media (Sunderland and Wicks, 1971) or by floating them on the surface of a liquid medium (Wernicke and Kohlenbach, 1976, 1977; Sunderland and Roberts, 1977). Directions for the preparation of the culture medium are given at the end of this section.

6. The anthers are cultured at 25°C in light or darkness. Light is essential after plantlet formation has been initiated for the production of chlorophyll and normal plant growth. Continuous illumination from cool-white fluorescent lamps (300 lux) gives satisfactory results (Kasperbauer and Wilson, 1979). Young embryos can be detected by gently bursting the cultured anthers on a microscope slide in a drop of acetocarmine stain. Plantlet formation occurs after a period of approximately 4–5 weeks of culture.

7. Separate the plantlets with forceps and discard the remaining anther tissue. In order to hasten the development of the plantlets, transfer them to a root-inducing medium after they are about 3 mm in length. This medium is identical to the anther culture medium, except that the agar is reduced to 0.5% (w/v) and all the other components are provided in half strength. During this period of growth the plants should be placed on a 12-hr daylength provided with 5,000 lux illumination from white fluorescent lamps (Kasperbauer and Wilson, 1979). It is recommended that the student examine the root tips of the plantlets with acetocarmine or Feulgen staining in order to verify the haploid chromosome number (Darlington and LaCour, 1976; Collins, 1979).

Experiment 2. Pollen culture (Sunderland and Roberts, 1977)

1–4. Same as the preceding experiment.

5. This technique is based on the release of pollen into the culture medium following the dehiscence of the tobacco anthers. Pretreatment of the tobacco buds by chilling (step 2) apparently facilitates the dehiscence. For each culture place the anthers from three tobacco buds in 5 cm^3 of liquid medium in a Petri dish. Remove and discard the anthers from some cultures after 6, 10, and 14 days. For anthers other than those of tobacco, it may be advisable to make a slight incision in the anther tissue with a fine scalpel blade and gently squeeze the contents of the anthers into the medium with forceps. Seal the dishes with Parafilm, and incubate them at 28°C in the dark for the first 14 days of culture. After

14 days transfer the cultures to an illuminated growth chamber (Gro-Lux fluorescent lamps, 500 lux, 12-hr daylength, 25°C). The pollen released from the anthers into the medium at 6-, 10-, and 14-day intervals will develop into haploid embryos (Sunderland and Roberts, 1977; Kasperbauer and Wilson, 1979; Nagmani and Raghavan, 1983).

Experiment 3. Regeneration of diploid plants

In this experiment an attempt will be made to promote chromosome doubling of the plantlets formed from the anther culture experiment. When plantlets are beginning to emerge from the cultured anthers, immerse the anthers in an aqueous solution of colchicine (0.5% w/v) for 24–48 hr. Colchicine is a powerful poison, and students should exercise the utmost care in handling this alkaloid (see appendix to Chapter 7). Following the colchicine treatment, rinse the plantlets in DDH_2O and culture them as described in experiment 1, step 7. It is important to ascertain the chromosome number of the plantlets with acetocarmine or Feulgen staining. If diploidy is not achieved, the immersion time may be increased to 96 hr, and it may be necessary to repeat the treatment several times in order to achieve success (Sunderland and Dunwell, 1977).

Preparation of anther and pollen culture media

The anthers of many plants, including *Nicotiana tabacum* and *Hyoscyamus niger*, can be cultured on relatively simple media consisting of minerals and sucrose. Other species, however, require the addition of organic

Table 14.1. *A basal medium for the liquid culture of pollen*

Constituent	Concentration (mg/l)
KNO_3	950
NH_4NO_3	825
$MgSO_4 \cdot 7H_2O$	185
$CaCl_2$	220
KH_2PO_4	85
$FeSO_4 \cdot 7H_2O$	27.8
Na_2EDTA	37.3
sucrose	20,000

Source: Sunderland and Roberts, 1977.

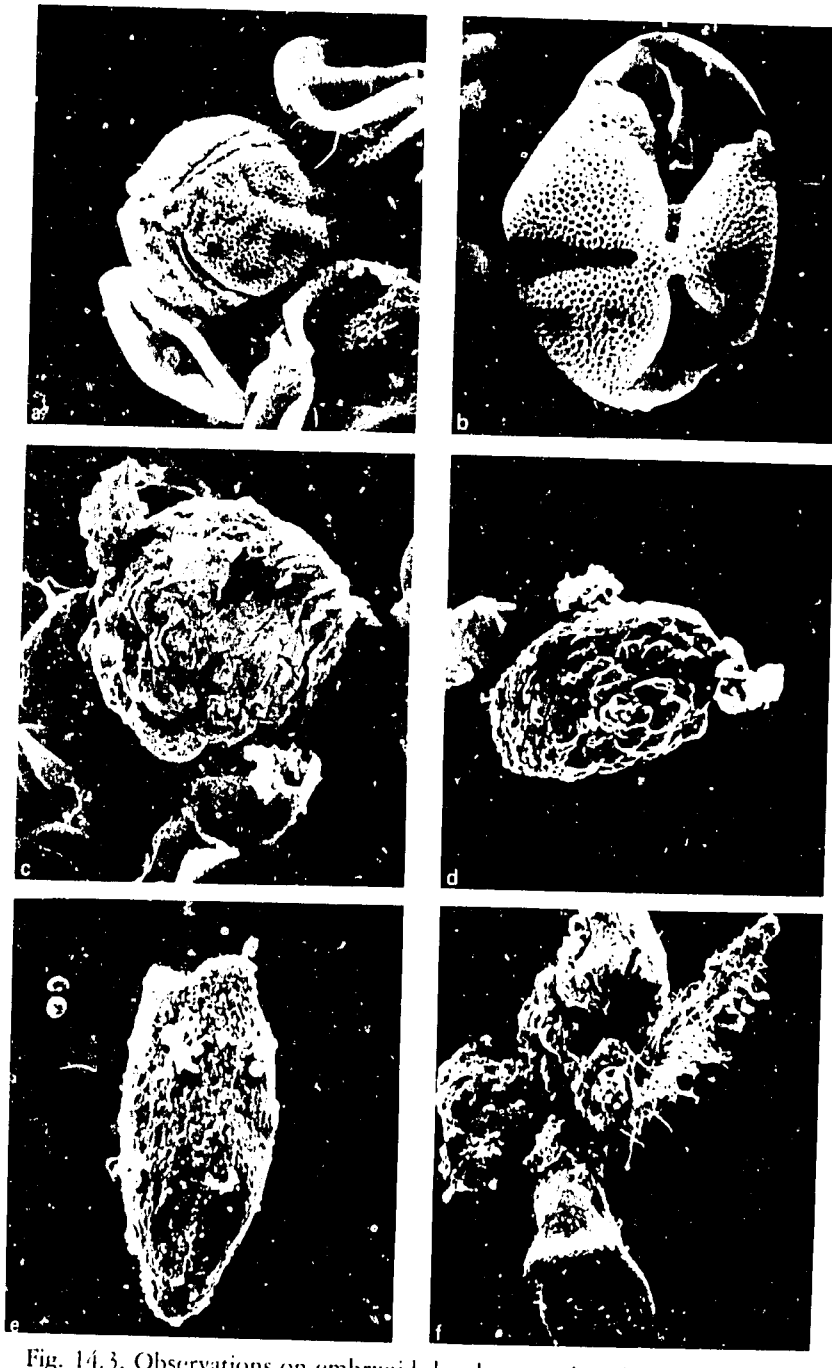


Fig. 14.3. Observations on embryoid development in cultured anthers of *Hyoscyamus niger* (henbane) with scanning electron microscopy. (a) Developing pollen grain after 20 hours culture. (b) Swollen grain beginning to break open at raphe after 5 days of culture. (c) Globular stage of embryoid after 7 days' culture showing association with the

supplements and plant hormones. Unfortunately, the addition of auxin to the culture medium appears to stimulate callus formation (Raghavan, 1978). The medium recommended for the culture of anthers in experiment 1 is the MS medium supplemented with 2% (w/v) sucrose. If desired, the medium may be solidified with agar (0.6–0.8% w/v).

It is suggested that the student employ an alternative medium, devised by Sunderland and Roberts (1977), for the liquid culture of isolated pollen. The composition of the medium is given in Table 14.1.

Preparation of acetocarmine stain

Acetocarmine is prepared by refluxing an aqueous solution of carmine (4% w/v) for 24 hr in acetic acid (50% v/v), and then filtering the product. Species with a low DNA content should first be stained with the Feulgen reaction. Anthers are fixed in acetic acid:ethanol (1:3 v/v) for several hours (4°C), passed through a graded ethanol series to water, and hydrolyzed in HCl (5 N) for 1 hr at room temperature. The root tips or spores may be stained with the Feulgen reagent for approximately 2 hr, and then squashed in acetocarmine (Sunderland and Dunwell, 1977).

RESULTS

Remove the anthers from the culture medium at regular intervals, and observe microscopically the development of the pollen grains. For ease of interpretation Fig. 14.3 shows the development of embryoids from *Hyoscyamus niger* pollen as viewed with the scanning electron microscope (Dodds and Reynolds, 1980). After 2 or 3 days in culture certain developmental changes can be detected (Fig. 14.3). Many of the pollen grains accumulate starch granules, others degenerate, and a small proportion of them divide and enter into the developmental pathway leading to the formation of haploid embryoids (Raghavan, 1975, 1978). The stages of embryoid development arising from cultured pollen grains are similar to the stages of zygotic embryo development normally found in diploid plants. The embryogenic pollen grains eventually rupture, and the developing system undergoes repeated cell divisions. This cellular

Fig. 14.3. *cont.*
parental pollen grain. (d) Another embryoid showing globular stage after 7 days of culture. (e) Heart-shaped embryoid after 9 days of culture. (f) Embryoid bursting through the anther wall after 14 days of culture; note the developing roots and shoots. (From Dodds and Reynolds, 1980.)

proliferation gives rise to a globular-stage embryo (Fig. 14.3c,d), which undergoes morphological changes to produce heart-shaped (Fig. 14.3e) and torpedo forms. Eventually the haploid embryoids break through the anther wall (Fig. 14.3f). This entire process takes about 14 days in *Hyoscyamus*, and 21–28 days in *Nicotiana*.

The plantlets can be dissected from the anthers and grown to maturity. As outlined in experiment 3, it is possible to treat these haploid plants in such a way that they develop into homozygous and fertile diploid plants. Haploid plants are sterile because they are unable to undergo reduction division in meiosis.

QUESTIONS FOR DISCUSSION

1. Haploid production by microspores offers certain advantages over other methods for the formation of haploid plants. Comment on this statement.
2. Offer some possible explanations why cultured anthers will permit pollen to develop into embryos, whereas cultured isolated pollen grains may not form embryos.
3. Of what importance are haploid plants to the plant breeder?
4. What are the three pathways that *Hyoscyamus* pollen grains may follow when placed into culture?

APPENDIX

1. Try screening anthers from several species of plants in an attempt to produce embryoids. Plant families that have yielded haploid plants from anther cultures include the Solanaceae, Gramineae, and Cruciferae. In addition to the plant species mentioned in this chapter, some other plants that may be useful include *Populus*, tomato (*Lycopersicon esculentum*), *Petunia hybrida*, maize (*Zea mays*), sugar beet (*Beta vulgaris*), and wheat (*Triticum aestivum*). Following plantlet formation, determine the percentage of haploids among the newly formed plants by using aceto-carmine squash preparations of the root tips.

2. With the aid of a hemocytometer, determine the number of pollen grains within a single anther taken from your experimental plant. What percentage of pollen grains formed embryoids from the anthers you cultured?

3. Repeat experiment 1 with the agar medium supplemented with activated charcoal (2% w/v). Do you find a greater percentage of anthers forming embryoids compared to the original results? Is the total number of plantlets produced greater or less in comparison to the medium lacking the charcoal?

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15

Storage of plant genetic resources

Considerable interest has been shown in recent years on the application of tissue culture technology to the storage of plant germplasm (Bajaj and Reinert, 1977; Wilkins and Dodds, 1983a,b). The conventional methods of germplasm preservation are prone to possible catastrophic losses due to: (1) attack by pest and pathogens, (2) climatic disorders, (3) natural disasters, and (4) political and economic causes. In addition, the seeds of many important crop plants lose their viability in a short time under conventional storage systems. Examples of plants bearing short-lived seeds include *Elaeis guineensis* (oil palm), *Hevea brasiliensis* (rubber), *Citrus* sp., and *Coffea* sp. Other reasons for using tissue culture procedures in the conservation of germplasm will be discussed.

Vegetatively propagated plants that have a high degree of heterozygosity or that do not produce seed must be stored in a vegetative form as tubers, roots, or cuttings. This usually involves high-cost labor during the growing season and, in addition, the field collection and storage of propagules for short periods of time. Crops that fall into this category include yams (*Dioscorea*), bananas (*Musa*), potatoes (*Solanum*), cassava (*Manihot*), taro (*Colocasia esculentum*), and sweet potato (*Ipomoea batatas*). An interesting example is the large-scale propagation of the international collection of potato germplasm at the International Potato Center (CIP), Peru, which in 1980 involved 12,000 accessions. Several of these potatoes are infertile polyploid cultivars for which seed storage is obviously impossible.

For many species, including coconut (*Cocos nucifera*), oil palm (*Elaeis guineensis*), and date palm (*Phoenix dactylifera*), there is no efficient method of vegetative propagation. The conventional method of reproduction of date palm, i.e., by suckers arising from the base of the main stem, is slow and unreliable. The development of a suitable tissue culture system of propagation and conservation of germplasm would therefore have obvious benefits. In the case of date palm the situation is especially

serious. The genomes of this species in Algeria and Morocco are bordering on extinction due to Bayoun disease (*Fusarium oxysporum* Schlect. var. *albedinis*).

Many long-lived forest trees, including angiosperms and gymnosperms, do not produce seed until a certain age, and these trees must be propagated vegetatively when it is necessary to produce the parental genotype. The most frequently used methods of vegetative propagation of rosaceous fruit trees are budding and grafting. One area of research currently under investigation is the possibility of rapid clonal propagation of fruit trees by means of tissue culture techniques. These techniques have special relevance to certain fruit-tree cultivars that are either difficult and/or expensive to propagate by conventional methods. For example, scions of apple cultivars are usually propagated by budding or grafting to rootstocks that are themselves raised by stooling or layering. This process takes three years and demands expensive nursery facilities and skills.

Propagation by the production of self-rooted plants would be much more rapid and could be of value in hastening the availability of new cultivars developed from breeding programs. An additional advantage of tissue culture techniques to fruit-tree propagation is the rapid in vitro multiplication and rooting of apple rootstocks such as M9. This rootstock is widely used because of its effects on precocity and on the control of tree and fruit size. A major disadvantage, however, is that cuttings are extremely difficult to root using conventional methods. Microprogation with in vitro techniques has also been applied to *Prunus* cultivars, thus making available large quantities of material for use in breeding programs (Jones, Pontikis, and Hopgood, 1979).

Cryopreservation. It will be evident from the previous sections that an in vitro system with a high multiplication rate, although ideal for purposes of clonal propagation, is entirely unsuitable as a means of germplasm conservation. Such systems require frequent attention and maintenance, and the mutation rate is likely to correspond directly to the rate of cell division (Henshaw, O'Hara, and Westcott, 1980). Consequently an ideal system for germplasm storage would be to store material in such a manner as to achieve complete cessation of cell division. This can be accomplished by storage of the plant material at the temperature of liquid nitrogen (-196°C). Although such techniques have been applied to a range of tissue cultures, the success rates have been variable. Some aspects of freeze- or cryopreservation are discussed by Withers (1980, 1982a, 1983b).

Minimal growth storage. Techniques of germplasm conservation based on the storage of shoot-tip cultures or meristem-derived plantlets under conditions that permit only minimal rates of growth will have widespread application in the near future. Such systems already have important uses in several international germplasm resource centers, mainly because the stored material is readily available for use, can be easily seen to be alive, and the cultures may be readily replenished when necessary (Wilkins and Dodds, 1983b).

There have been several approaches to growth suppression in plant tissue cultures. Three principal methods are used. (1) The physical conditions of culture can be altered, e.g., temperature or the gas composition within the culture vessels. (2) The basal medium can be altered, for example, using sub- or supraoptimal concentrations of nutrients. Some factor essential for normal growth may be either omitted or employed at a reduced level. (3) The medium can be supplemented with growth retardants (e.g., abscisic acid) or osmoregulatory compounds such as mannitol and sorbitol.

Reviews of tissue culture conservation via minimal growth techniques have been given by several workers (Withers, 1980; Henshaw et al., 1980). Several aspects of these techniques with the potential for immediate application will be discussed.

Temperature reduction has been very effective as a means of storage of tissue cultures. Some of the many species that can be conserved by this method include grapes (*Vitis rupestris*), potatos (*Solanum*), beets (*Beta vulgaris*), apples (*Malus domestica*), strawberries (*Fragaria vesca*), sweet potato (*Ipomoea batatas*), cassava (*Manihot esculentum*), and various forage grasses (e.g., *Lolium, Festuca, Dactylis*, and *Phleum*). In all cases the storage temperature employed is dependent on the particular crop species. As a general rule, temperate crops such as potatoes, strawberries, grasses, and apples are stored at 0–6°C, whereas tropical crops, such as cassava and sweet potato, are stored within the range 15–20°C.

For certain species the use of a reduced-temperature storage system has proved very successful. Meristem-derived plantlets of strawberry have been stored for as long as six years. It is not surprising that this technique is currently used on a routine basis at two international genetic resource centers.

The use of a decreased atmospheric pressure and a lowered partial pressure of oxygen has been proposed as a means of minimal growth storage (Bridgen and Staby, 1983). The addition of growth retardants to culture media as a means of inducing minimal growth has been attempted

by several workers. The compounds employed have included abscisic acid, mannitol, sorbitol, tributyl-2,4-dichlorobenzylphosphonium chloride (Phosfon D), maleic hydrazide, succinic acid-2,2-dimethyl hydrazide (B-995), (2-chloroethyl)-trimethylammonium chloride (CCC), and ancymidol. The effects of some of these growth retardants on the survival of potato cultures were given by Westcott (1981). The survival of potato cultivars after 12 months' growth in the presence of these inhibitory substances was roughly equivalent to the results obtained by using reduced temperatures and decreased levels of nutrients (Westcott, 1981). A choice of storage methods is therefore available.

Investigations at the University of Birmingham have shown that cultures of temperate fruit trees may be stored for several months by various techniques. Cherry-shoot cultures have survived on a liquid medium at normal temperatures for prolonged periods (Wilkins, Bengochea, and Dodds, 1982). Similar unpublished results have been obtained by this research group with cultures of apple, plum, and pear cultivars.

In the present experiments shoot tips of potato will be conserved by freezing in liquid nitrogen and by culture on a medium containing maleic hydrazide as a growth inhibitor.

LIST OF MATERIALS

Sterilization mode: C, chemical; O, oven; A, autoclave

shoot-apex culture of potato exhibiting regeneration (see chap. 10)
C scalpel
O forceps
O Petri dishes, 100 × 15 mm (five)
O culture tubes (30)
O aluminum foil (cut into squares)
A 200-cm^3 potato shoot micropropagation medium (see chap. 10; sufficient amount for 20 culture tubes)
A 100-cm^3 potato shoot micropropagation medium supplemented with maleic hydrazide (10 mg/l) (employ 10 culture tubes)
ethnol (80% v/v) dip
ethanol (70% v/v) in plastic squeeze bottle
Dewar flask of liquid nitrogen
face mask (protective plastic)
asbestos gloves
long forceps, approximately 30–40 cm in length
plant growth chamber (illuminated; 25°C)

PROCEDURE

Experiment 1. Storage of shoots in the presence of growth retardant

1. Actively propagating potato shoots (see chap. 10) are removed from the culture vessel and transferred to sterile Petri dishes for dissection of the individual shoots.

2. Using sterile instruments 10 shoots are transferred to culture tubes containing a fresh micropropagation medium as a control, and 10 shoots are transferred to tubes containing a similar medium supplemented with maleic hydrazide (10 mg/l).

3. The cultures are incubated in illuminated plant growth chambers (25°C). Measurements are made of the growth of the cultures at monthly intervals.

Experiment 2. Freeze preservation of potato shoot tips

1. Excise some sterile potato shoot apices (see chap. 10). Wrap them carefully in sterile squares of aluminum foil.

2. While wearing protective gloves and a face mask, plunge the foil packet into a Dewar flask of liquid nitrogen (−196°C). Permit the packet to remain in the liquid nitrogen for several minutes. The sample can be left for longer periods of time if a liquid-nitrogen storage tank is available.

3. Remove the foil packet and allow it to warm to room temperature.

4. Open the packet and carefully transfer aseptically the frozen and thawed apices to culture tubes containing the potato shoot micropropagation medium (10 tubes).

RESULTS

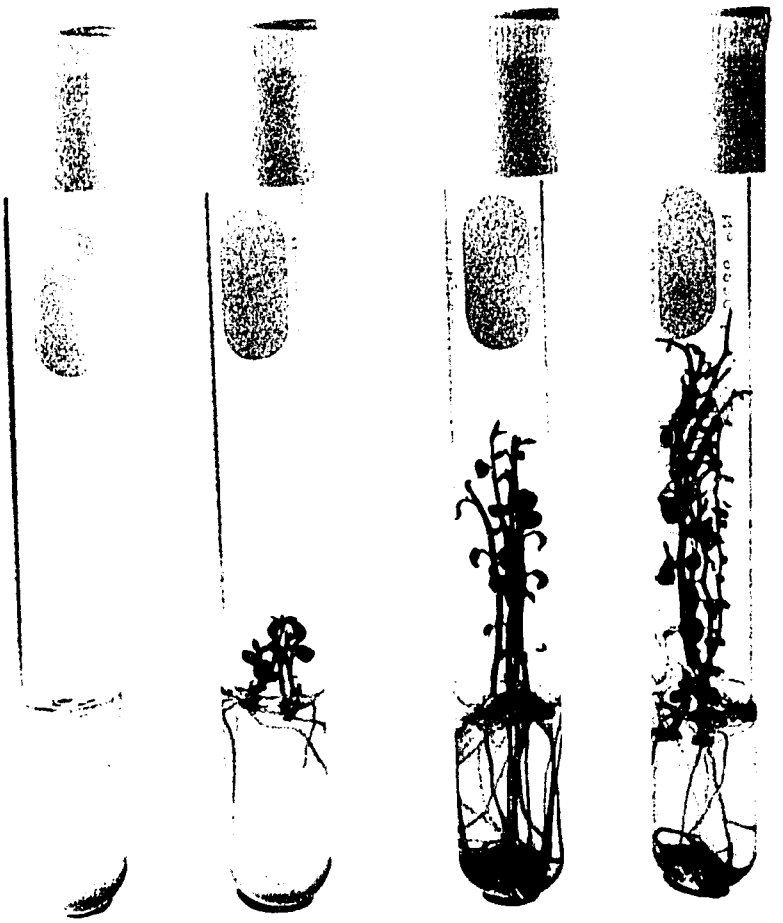
Experiment 1

The control shoots transferred to the normal micropropagation medium grow rapidly under these culture conditions. The growth retardant has a severe effect on shoot growth. A comparison of growth retardant to control cultures following a six-week period is shown in Fig. 15.1.

Experiment 2

The time required for excised shoot apices to initiate growth varies greatly, but growth should be easily visible after 4–6 weeks with potato.

Fig. 15.1. Effect of the growth retardant maleic hydrazide on in vitro cultures. Left-hand tube shows freshly transferred culture and right hand shows control culture after six weeks. The intermediate culture tubes contain 10 and 1 mg/l of maleic hydrazide.



The success rate or percentage of survivors will be relatively low ar the results will vary from one species to another.

QUESTIONS FOR DISCUSSION

1. Why are tissue cultures used to conserve germplasm of plants?
2. Name some plants in which tissue culture is the only method for germplas conservation.
3. What techniques are available for in vitro conservation? Discuss the adva tages and disadvantages of each technique.

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16

Production of secondary metabolites by cell cultures

Aside from the primary metabolic pathways common to all life forms, some reactions lead to the formation of compounds unique to a few species or even to a single cultivar. These reactions are classified under the term "secondary metabolism" and their products are known as "secondary metabolites" (Luckner and Nover, 1977). These substances include alkaloids, antibiotics, volatile oils, resins, tannins, cardiac glycosides, sterols, and saponins. In addition to their economic importance, many secondary metabolites play ecological and physiological roles in higher plants. Investigations in the area of biochemical ecology indicate that some secondary compounds produced by plants are important either to protect these plants against microorganisms and animals, or to enhance the ability of one plant species to compete with other plants in a particular habitat (Bell, 1980). For additional background, Mothes (1980) has given an excellent introduction to secondary plant products.

Despite advances in the field of organic chemistry, plants are still an important commercial source of chemical and medicinal compounds. The chief industrial applications of secondary metabolites have been as pharmaceuticals (e.g., sterols and alkaloids), and as agents in food flavoring and perfumery (Collin and Watts, 1983). In some cases, these plants have not been subjected to intensive genetic programs for the optimum production of the compound. In addition, there have been technical and economic problems in the cultivation of these plants. Unfortunately, many Third World countries producing medicinal plants are politically unstable, and the supply of crude plant material for processing cannot be guaranteed.

It has been proposed that many of these secondary metabolites produced by intact plants could be synthesized by cell cultures (Klein, 1960; Puhan and Martin, 1970). The basic technology involved in submerged cell cultures on a large scale was described by Nickell (1962). Several patents have been obtained for the production, from cell cultures, of such

metabolites as allergens, diosgenin, L-dopa, ginseng saponin glycosides, and glycyrrhizin (Staba, 1977). Tissue cultures have produced compounds previously undescribed, and cultures of higher plant cells may provide an important source of new economically important compounds (Butcher, 1977).

Although the production of secondary metabolites by cell cultures may be impractical, in some cases, the techniques of plant tissue culture can be used to improve the cultivation of these plants. These culture procedures include vegetative propagation, the isolation of virus-free stock, mutation studies with haploid plants, protoplast fusion, and the screening of disease-resistant lines.

There are numerous reasons why progress has been slow in the industrial application of cell cultures for the production of secondary metabolites. The cultures exhibit relatively slow rates of growth, and the biosynthesis of the desired compounds is often at a much lower level than in the intact plant. In order for cell cultures to be used as commercial sources of these compounds, the in vitro production must be comparable to or exceed the amount produced by the intact plant. Several reports have been published indicating yields approaching or exceeding yields from the whole plant (Table 16.1). In some cases, the production of

Table 16.1. *Secondary metabolites produced in cell cultures at levels equal to or exceeding those found in the intact plant*

Compound	Plant species	Reference
nicotine	*Nicotiana rustica*	Tabata and Hiraoka, 1976
serpentine	*Catharanthus roseus*	Döller et al., 1976
anthraquinones	*Morinda citrifolia*	Zenk et al., 1975
diosgenin	*Dioscorea deltoidea*	Kaul et al., 1969
thebain	*Papaver bracteatum*	Kamimura et al., 1976
proteinase inhibitors	*Scopolia japonica*	Misawa et al., 1975
ginseng saponins	*Panax ginseng*	Jhang et al., 1974
phenolics	*Acer pseudoplatanus*	Westcott and Henshaw, 1976
flavonols	*Acer pseudoplatanus*	Westcott and Henshaw, 1976
coumarin derivatives	*Ruta graveolens*	Steck et al., 1971
alkaloids	*Ruta graveolens*	Steck et al., 1971
solasadine	*Solanum lacinatium*	Chandler and Dodds, 1983a,b

secondary metabolites does not show a positive correlation with the maximal growth rate of the culture. This observation may reflect a competition for metabolites utilized in primary metabolism with those pathways leading to the formation of secondary products; for example, a competition could exist for amino acids in the formation of proteins, alkaloids, and phenylpropanoids (Aitchison, Macleod, and Yeoman, 1977). One approach used to regulate metabolic pathways favoring the production of secondary metabolites has been to add precursors to the culture medium. Although enrichment of the medium with precursors has given some degree of success, failure of the desired result may be due to lack of uptake, precipitation, conjugation, diversion into alternative pathways, or lack of one of the enzymes between precursor and product (Aitchison et al., 1977). Attempts have been made to increase the yields of cell cultures by selecting high-yielding strains (Tabata et al., 1978). Although the biosynthesis of secondary metabolites is not accompanied by visible markers, radioimmunoscreening methods have been developed for cell cultures producing secondary metabolites (Weiler, 1977). The instability of cell cultures for the continued production of secondary products poses a problem, since some cell lines lose the ability to synthesize the desired compound after prolonged culture. Obviously, it is important to examine thoroughly the continuing production of a given strain before scaling up for industrial production (Alfermann and Reinhard, 1978). Finally, there is the high cost involved in the large-scale production of cell cultures (e.g., in glassware, chemicals, and technicians). With the present level of development it appears that only rare and very expensive chemicals can be commercially produced by this method (Zenk, 1978).

The relationship between the degree of tissue organization and the biosynthesis of secondary products is obscure. The spatial orientation of enzymes, compartmentalization of enzymes and substrates, and reservoir sites for product accumulation may be some of the factors involved in the biosynthesis of secondary products by specialized tissues (Butcher, 1977). The metabolic requirements for some of these biosynthetic pathways, however, do not depend on the level of cytodifferentiation. In a review on secondary-product formation in cell cultures, Butcher (1977) has subdivided these compounds into four general groups.

1. Some compounds occur throughout the plant kingdom and are not associated with any level of differentiation (e.g., phytosterols and certain flavonoids).
2. Some widely distributed compounds are restricted to certain types (e.g., lignins and tannins).
3. Some compounds are restricted to certain plant families and species, although

the biosynthesis is not associated with any form of cytodifferentiation (e.g., specific flavonoids and anthraquinones).

4. The biosynthesis of some compounds is restricted to highly specialized cells or tissues (e.g., essential oils, resins, and latex). Within this group the level of differentiation is directly related to the biosynthesis of the compound.

In the last category, we can assume that progress toward inducing certain levels of cytodifferentiation in cell cultures must be made before success will be achieved in the in vitro biosynthesis of these secondary metabolites (Street, 1977; Yeoman et al., 1982).

Discussions of the possible relationships between secondary metabolism and cytodifferentiation have been published (Böhm, 1977; Yeoman et al., 1982). The main problem in establishing these relationships, as in most cell culture research, is that the method of culture is empirical and cannot be transferred from one plant species or variety to another.

Because the principles of chemical biosynthesis by cell cultures are poorly understood, we cannot anticipate a quick success as far as practical applications are concerned (Alfermann and Reinhard, 1978). Yet, in spite of numerous problems, there are active research programs on the in vitro production of secondary metabolites, particularly in Ireland, Japan, West Germany, Israel, India, and the United States.

Several secondary metabolites produced by cell cultures are pigmented (Reinert, Clauss, and Ardenne, 1964; Alfermann and Reinhard, 1971; Strickland and Sunderland, 1972). In the present chapter the student will study the in vitro biosynthesis of secondary metabolites either by a visual examination of cultures for anthocyanin pigment formation or by the detection of aromatic compounds in mint cultures (Lin and Staba, 1961). The aromatic monoterpenes produced by mint can be detected by smelling the cultures or by gas–liquid chromatography, if available.

LIST OF MATERIALS

Sterilization mode: C, chemical; O, oven; A, autoclave

mature plants of *Mentha, Haplopappus*, or *Daucus carota* 300 cm^3 aqueous solution (10% v/v) commercial bleach containing 2 cm^3 of a wetting agent (1% v/v)
paring knife
C scalpel
O forceps
O 9-cm Petri dishes for explant culture (12)
O 9-cm Petri dishes for explant preparation and rinsing (five)

O stainless steel cork borer (no. 2) containing metal rod
A 9-cm Petri dishes containing two sheets of Whatman No. 1 paper (five)
A explant cutting guide
A 400 cm^3 MS medium for callus induction (see chap. 4 and 5)
A 125-cm^3 Erlenmeyer flasks containing 100 cm^3 DDH_2O (12)
O 600-cm^3 beakers (four)
ethanol (80% v/v) dip
ethanol (70% v/v) in plastic squeeze bottle
methanol lamp
interval timer
heavy-duty aluminum foil (one roll)
incubator (26–28°C)

Leaf or stem material of *Mentha* can be employed for the stud aromatic compounds. Stem material of *Haplopappus* and taproot of *D cus carota* will yield explants suitable for the production of anthocy pigments.

PROCEDURE

1. Suitable plant material is surface sterilized according to the p cedure given in Chapter 5. Explants of the tissue, rinsed in DDH_2O blotted on Whatman No. 1 filter paper, are placed on the culture med in order to initiate callus. The cultures are incubated at 26–28°C in presence or absence of artificial light.

2. After 3 weeks the callus may be subcultured to a freshly prepa medium.

Anthocyanin pigment formation. Following development of callus fr the *Haplopappus* or *Daucus* explants, some areas of the callus will white, and other areas will be pigmented. Fragments of callus exhibit varying degrees of pigmentation should be carefully excised from t callus mass and subcultured separately on a fresh medium. In this v it is possible to build up a number of "cell lines" with different anth cyanin pigment characteristics. This selection process is time-consumi and a minimum of several months will be required in order to devel a collection of cell lines.

Aromatic terpenes. After callus has been initiated from the *Mentha* explan it should be subcultured on a fresh medium. Small portions of the call can be "sniffed" to see if they still possess the characteristic mint odc

If gas–liquid chromatography is available, transfer a fragment of callus to a culture tube sealed with a gas-tight fitting. After a few hours remove a gas sample from the culture tube with a hypodermic syringe. Apply the gas sample to the GLC instrument (flame ionization detector; 6′ × 1/8″ 15% DEGS-filled glass column; 40 cm^3/min N_2 and H_2 flow rate; injector, detector, and column temperatures adjusted to 180°, 180°, and 130°C, respectively.) For additional information, see the procedure outlined by Aviv and Galun (1978).

RESULTS

Anthocyanin pigments. As callus is initiated from the *Haplopappus* and *Daucus* explants, localized areas of pigmentation occur. Some of these areas are completely white, whereas others have an orange or pink coloration. When small inocula are removed from the callus mass and subcultured, each cell line maintains its own distinctive coloration.

Aromatic terpenes. In the early stages of callus formation and during the first few subcultures, the odor of the callus clearly indicates the presence of the characteristic monoterpenes. As the callus is repeatedly subcultured, the levels of aromatic compounds gradually decline.

QUESTIONS FOR DISCUSSION

1. Name some important pharmaceutical chemicals produced by plants.
2. What are the advantages of producing pharmaceutical compounds from cell and tissue cultures?
3. What is the genetic significance of cell-line production of anthocyanin pigments? Are the cell lines genetically comparable?

APPENDIX

Two additional experiments can be conducted on the production of secondary metabolites by callus cultures.

1. Subculture callus to an MS medium in which the mineral salts have been diluted by a factor of 10 (i.e., 1 part MS salts: 9 parts DDH_2O). In comparison with the original experiment, is the concentration of secondary products higher or lower?

2. Allow the callus culture to be maintained on the same medium without subculturing for approximately 6 weeks. Does prolonged culture on the same medium influence the production of secondary products?

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17

Quantitation of tissue culture procedures

The results of experiments conducted on plant tissue cultures are expressed in qualitative descriptions as well as in measurements of a quantitative nature. The growth of a culture over a period of time, whether it is a callus or a suspension culture, is characterized by an increase in cell number, an increase in volume or mass, and changes in biochemistry and cellular complexity.

Sorokin (1973), in a discussion of the terminology of growth in connection with algal suspensions, raised some interesting points that can be applied to suspension cultures of higher plant cells. Because the term "growth" is ambiguous, referring to both the product of the process and the process itself, it is preferable to designate the product as "yield" and reserve "growth" for the process of accumulation of the product. Yield does not represent "total growth" of the system, but only the net result of the metabolic processes (anabolic gain, catabolic loss). There will be losses also due to cell death. The expression of yield must be accompanied by the time period involved. In the case of batch cultures of cell suspensions, yield is determined at the cessation of growth or at the beginning of the stationary phase.

A wide choice of parameters can be employed in the analysis of growth rates. In this brief introduction to quantitative analyses, we will consider a few of the basic techniques of measurement that may be applied to root (R), callus (C), and suspension (S) cultures as shown in Table 17.1.

The simplest measurement involves the increase in length of cultured roots (see chap. 8). Periodic measurements can be made with the metric rule held beneath the growing roots in the culture flasks or by the use of graph paper placed beneath the cultures. Sterility of the cultures is not endangered. Because cultured roots normally do not exhibit secondary thickening, the cross-sectional area remains constant during growth. Therefore increments in length represent an accurate measure of volume change with growth (White, 1963). Although this method

ignores branch root formation, the error introduced in the measurement over a 7-day period is never beyond 10% and seldom exceeds 1% (White, 1943).

Although the growth rates of callus cultures are frequently expressed on the basis of increases in fresh weight, these values should not be used in the estimation of cell numbers and cell division rates. Callus is heterogeneous, and the callus mass has centers of high division rates and regions of low metabolic activity. It is advantageous to measure fresh weight as a growth parameter because this is a rapid method of following an increase in tissue mass. Although it is possible to weigh callus samples periodically under aseptic conditions, the student will find that this practice almost invariably leads to some degree of contamination. The fresh weight of a cell suspension can also be determined by collecting the cells on a filter of industrial nylon mesh. Directions for this procedure will be given at the end of the chapter.

The measurement of the dry weight of a callus gives an acceptable estimation of the biosynthetic activity of a culture; and, at fresh weights below 500 mg, the relationship between fresh and dry weight is approximately linear (Wetter and Constabel, 1982). One possible difficulty is the accumulation of large amounts of carbohydrates within the cells, which tends to complicate the interpretation of the data (Yeoman and Macleod, 1977). The dry weight of the callus at the termination of the experiment minus the dry weight of the primary explant, at the beginning

Table 17.1. *Various techniques for the measurement of growth and differentiation in plant tissue cultures*

Technique	Application[a]
linear growth rate	R
fresh weight	C, S, R*
dry weight	C, S, R*
density cell count	S
total cell count	C
packed cell volume	S
mitotic index	S, R, C*
tracheary element count	C, S*

[a] The applications refer to cultures of root (R), callus (C), and suspensions (S). The asterisk indicates that the technique applies to a lesser extent.

of the experiment, gives the increase in dry weight per unit of time. Obviously "the dry weight of the explant" does not refer to the explant that is actually cultured! A mean dry weight is obtained from several explants of the same dimensions as the cultured explants. Drying the sample should be continued until two successive weighings of the sample and tray give a constant weight; prolonged drying causes oxidative changes in cell dry weight in addition to water loss. The dry weight of a cell suspension can be determined by collecting the cells on a preweighed nylon filter. Cell suspension fresh and dry weights are normally given per cubic centimeter of culture and per 10^6 cells (Street, 1977)

Another procedure, used in connection with suspension cultures, measures the total amount of cell mass in a given volume of culture by compressing the cells to a constant volume with centrifugation. The result is known as the packed cell volume (PCV). A known volume of the suspension is transferred to a graduated conical centrifuge tube. For greater accuracy, a special centrifuge tube terminating in a calibrated capillary tube is recommended. The latter vessel, employed in the measurement of the PCV of algal cultures, accepts a 4–7 cm^3 sample and has a capacity of 50 μl in the calibrated section of the tube. The scale is subdivided into units of 1.0 μl, and estimations can be made to 0.1 μl (Sorokin, 1973). The centrifugation conditions may vary with different cultures. Although Street (1977) recommends a centrifugation of 2,000 × for 5 min for suspension cultures, a force of 1,500 × for 30 min is recommended to pack a *Chlorella* sample to constant volume (Sorokin, 1973). The optimum conditions can be determined by taking a reading after a trial run, giving the sample an additional period of centrifugation, and making a second reading. The two readings should be the same. The main sources of error in the determination of PCV are (1) poor sampling of the original culture, (2) inadequate centrifugation, and (3) a delay in reading PCV, which leads to swelling of the cells and inflated values (Sorokin, 1973).

One important indication of growth is the increase in cell number of the culture. In agar cultures estimates can be made of the total cell number of the primary explant and accompanying callus. The cell count in suspension cultures is expressed as density or concentration of cells per cubic centimeter of culture. The main problem with cell-counting methods is the separation of the cells into a homogeneous unicellular sample. Moreover, the approach is largely empirical because cultures differ widely in their response to cell separation treatments. Many laboratories have used some modifications of the Brown and Rickless (1949) technique of macerating the cells with chromium trioxide or a mixture of

chromium trioxide and hydrochloric acid. (An aqueous solution of chro mium trioxide is chromic acid.) Although this is probably the best metho of cell separation, care must be taken to avoid prolonged treatment lead ing to cell destruction. Some cultured tissues, however, will separate int individual cells following treatment with EDTA (Phillips and Dodds 1977) or pectinase (Street, 1977). Following separation, a known volum of the sample is placed on a hemocytometer slide or a Sedgwick-Rafte slide for counting. A mean of 10 field counts (100 × magnification) is obtained, and the total number of cells in the sample is then calculated. A discussion of the statistics of sampling with the Sedgwick-Rafter chamber for phytoplankton cell counts is given by McAlice (1971).

The phenomenon of xylogenesis can be quantitated in an explant by counting the numbers of tracheary elements in a sample following cell separation by a suitable technique. The details of this method will be given in the procedure section of this chapter.

The mitotic index (MI) represents the percentage of the total cell population of a culture that, at a given time, exhibits some stage of mitosis. Rapidly growing cell populations exhibit an MI of approximately 3–5% (Wetter and Constabel, 1982). Although the MI is a useful technique for studying the growth of a culture, many factors influence its value. Among these factors are the time required for the completion of a cell cycle, the duration of mitosis, the percentage of noncycling and dead cells, and the degree of division synchrony of the cell population. Because of the involvement of these factors, the determination of the MI alone is not an accurate reflection of the division synchrony of a given culture. A better approach for appraising the degree of division synchrony of a culture is to combine data from three sources: (a) measurement of the MI, (b) cell numbers showing the extent of synchrony of cytokinesis, and (c) degree of S-phase synchrony with tritiated thymidine. Several studies have been made on the synchrony of division in actively dividing calius and suspension cultures involving the calculation of the MI (Yeoman, Evans, and Naik, 1966; Yeoman and Evans, 1967; Street, 1968). The procedure is tedious because at least 500 nuclei should be examined for signs of mitosis. The number of samples prepared for each callus growth is empirical, and it is impractical to measure the MI of large fragments of callus. In mature callus a relatively small proportion of the cells will be actively dividing, and these meristematic cells will be localized in small growth centers or cambial zones. The sampling is more suitable for cell suspensions than callus. Of the several nuclear staining methods that have been used, the present experiment will employ the

carbol-fuchsin stain of Carr and Walker (1961) as modified for plant cells (Kao 1982). Alternative staining procedures are given in Street (1977) and Yeoman and Macleod (1977).

LIST OF MATERIALS

Experiments 1, 2. Determination of fresh and dry weight

equipment for initiation of callus (see chap. 5)
fresh plant material for the preparation of noncultured explants
aluminum foil weighing trays. If dry weight determinations are to be made with cell suspension cultures, nylon mesh, cut into discs, will be necessary to collect the cells.
9-cm Petri dishes, each containing two sheets of Whatman No. 1 filter paper (five)
desiccator containing silica gel
analytical balance
drying oven (60°C)

Experiment 3. Determination of culture density by cell count

suspension culture (see chap. 9)
aqueous solution (8% w/v) chromium trioxide
laboratory oven (70°C)
bench-top shaker
vials for maceration; Pasteur pipettes
Sedgwick-Rafter chamber or hemocytometer slide
Whipple disc
stage micrometer slide
compound microscope (100 × magnification)
microscope slides; cover slips
calculator, hand model; hand tally (optional)

Experiment 4. Determination of packed cell volume (PCV)

suspension culture (see chap. 9)
bench-top centrifuge (set at 2,000 × g)
1-cm^3 pipettes
graduated conical centrifuge tubes, preferably terminating in a graduated capillary tube

Experiment 5. Determination of mitotic index (MI)

suspension culture (see chap. 9)
basic fuchsin
ethanol (70% v/v)
ethanol (95% v/v)
phenol (5% w/v)
acetic acid (45% v/v)
glacial acetic acid
formaldehyde (37% v/v)
sorbitol
5-cm^3 pipette, wide mouth
Pasteur pipettes
microscope slides; cover slips; glass rod
absorbent paper
10-cm^3 vials, with caps
compound microscope
hand tally (optional)

Experiment 6. Tracheary element counts in primary explants

primary explants cultured on xylogenic medium (see chap. 7)
maceration reagent consisting of 1:1 mixture of chromium trioxide (5% w/v) and hydrochloric acid (5% v/v)
Pasteur pipettes
10-cm^3 vials, with caps
syringe; no. 22-gauge and no. 18-gauge needles
volumetric tube, 2 cm^3 calibration mark
Sedgwick-Rafter chamber, cover slip
Whipple disc
compound microscope; green filter (optional)
calculator, hand model
hand tally (optional)

PROCEDURE

Experiment 1. Determination of fresh weight

1. Select suitable plant material for the induction of callus, and follow the instructions given in the Chapter 5 procedure. For example, lettuce pith, taproot of carrot, or soybean cotyledon would be ideal material for this experiment. It is suggested that the same explants be used for both

experiments 1 and 2; that is, after determination of the fresh weight of each explant, the same explant can then be oven dried. A total of 30 explants will be required (10 explants for each of the three sampling periods).

2. Either before or after the culture procedure, prepare at least 10–15 additional explants identical to the explants for culture. These "noncultured" explants need not be prepared under aseptic conditions.

3. Following a brief rinse in distilled water, place the noncultured explants on a double layer of Whatman No. 1 filter papter in a Petri dish. Cover the dish to prevent excessive water loss by the explants.

4. Weigh the blotted explants individually on a preweighed aluminum tray. They should be weighed on an analytical balance to the nearest 0.1 mg. Obtain a mean value for the gross weight (explant plus tray). Finally, subtract the weight of the tray from the mean value of the noncultured explants. These noncultured explants can be oven dried and the determinations used in experiment 2. See the directions given in experiment 2.

5. Weigh and sacrifice 10 cultured explants exhibiting callus growth after varying time periods (e.g., 7, 14, and 21 days). Transfer the callus samples from the culture medium to a Petri dish containing filter paper prior to placing them in the weighing tray. Remove any traces of the agar medium that may be clinging to the underside of each sample. Carefully wipe the surface of the weighing tray after each weighing to remove traces of residual moisture. Handle the tray with forceps because moisture from the fingers will alter the weight.

6. After obtaining the gross fresh weight (sample plus tray), subtract the weight of the tray from the net weight of the sample. Obtain a mean value of the fresh weights of the 10 samples. Subtract the mean value of the noncultured explants from the mean value of the sample fresh weight for each of the time periods (7-, 14-, and 21-day intervals). The latter values represent the fresh weight gain.

7. Plot the mean gain in fresh weight as a function of time.

Experiment 2. Determination of dry weight

The explants cultured in experiment 1 can be used for the dry weight determination of this experiment. If an additional culture is necessary for this experiment, use the same plant material and culture medium as employed in the previous experiment. A comparison of the fresh and dry weight increments, under the same cultural conditions, can then be made.

1. The directions for this experiment parallel, to some extent, the previous one. Explants, cultured on a callus-inducing medium, will be sacrificed at 7-, 14-, and 21-day intervals (10 explants for each time period). Place each callus sample in a preweighed aluminum foil weighing tray. Then place the tray and sample in a Petri dish and dry at 60°C for 12 hr. Cool the sample to room temperature in a desiccator containing silica gel, and weigh the sample (plus tray). Return the sample to the drying oven for an additional 4 hr, and repeat the process. If the sample was dried to constant weight, the two weighings will be the same. Subtract the dry weight of the tray from the preceding values to obtain the dry weight of the sample alone. Obtain a mean value of the dry weights of the 10 samples taken at each time interval.

2. The noncultured explants prepared in experiment 1 are oven dried and weighed in the same manner as the callus samples. In a similar manner the explants must be reweighed until a constant dry weight is obtained. Determine the mean value of the dry weight of the 10 samples of non-cultured explants. Subtract the latter value from the mean of the callus sample dry weight for each of the time periods. These values represent the gain in dry weight after 7, 14, and 21 days of growth.

3. Plot the mean gain in dry weight as a function of time on the same graph prepared for the fresh weight in experiment 1. Are the fresh weight and dry weight increments parallel over the 21 days of culture?

Experiment 3. Determination of culture density by cell count

The following two experiments are devised to measure the growth rate of a suspension culture by determining the density or number of cells per cubic centimeter of culture and the packed cell volume (PCV). A density cell count was conducted earlier in preparing the inoculum for a suspension culture (see chap. 9, Procedure).

1. Prepare in advance a suitable suspension culture as outlined in Chapter 9. The culture should be in a state of active cell division and consist primarily of single cells and small clumps with a relatively low level of lignification.

2. In order to separate the cell aggregates it is necessary to treat a sample of the suspension culture with chromium trioxide. It is suggested that the initial trial involve a mixture consisting of 5 cm^3 of culture plus 10 cm^3 of chromium trioxide (8% w/v). Heat the mixture to 70°C for 2 min, cool it to room temperature, and shake the mixture vigorously

on a bench-top shaker for approximately 10 min (Street, 1977). Place a droplet of the mixture on a microscope slide, and examine it for evidence of cell separation. If cell aggregates are still present, return the 15-cm^3 mixture of cells and chromium trioxide to the 70°C oven for an additional 5–10 min. Another maceration reagent consists of a mixture of equal parts chromium trioxide (10% w/v) and HCl (10% v/v); it can be used in a 1 : 1 ratio with the culture. It is important that a homogeneous single cell sample be taken of the suspension culture and that the culture be *thoroughly* agitated at the time of the removal of the sample by pipette.

3. Various types of hemocytometer slides can be used, although nearly all of these devices have very small volumes for plant cell counting. Possibly the best aid is the Sedgwick-Rafter slide that was originally devised for counting aquatic microorganisms. This chamber (50 × 20 × 1 mm) has an area of 1,000 mm^2 and a total volume of 1.0 cm^3. Because the chamber does not have grid markings, it is advisable to use a Whipple disc, which subdivides the optical field into squares. The Whipple disc is mounted inside the ocular of the microscope. In preparation for counting, it is necessary to determine the area covered in one optical field, that is, the field of vision seen under the Whipple disc at approximately 100 × magnification (10 × ocular and 10 × objective). The diameter of the field is measured with a stage micrometer, and the area is calculated. Next decide on a system for selecting 10 positions in the chamber to be counted (e.g., you might count 5 different positions across the center of the chamber and another 5 positions along the periphery). It will be necessary to refocus the microscope at each position in order to count all the cells because the cells will be in different planes of focus. Obtain a mean value for the number of cells in the 10 Whipple fields.

4. The calculations are made as follows. The area of the chamber is 1,000 mm^2. If the area of the Whipple field is 1.86 mm^2, there are a total of 537.6 Whipple fields in the entire chamber (1,000/1.86). Let N represent the mean cell count per Whipple field. The density (d) of the culture, given in cells per cubic centimeter, will be:

$$d = 537.6\,(N)$$

If the mean cell count per field is 15 cells, then the culture density is equal to 8,064 cells per cubic centimeter. If the sample has been diluted prior to counting, the density is then equal to $537.6(N)(DF)$. Dilution of the sample with water (1 : 1) gives a dilution factor (DF) equal to 2.

Experiment 4. Determination of packed cell volume (PCV)

1. Pipette from the suspension culture a known volume to be sampled, and transfer it to a graduated conical centrifuge tube. More accurate results may be obtained with a tube consisting of an enlarged upper part, the receiver, and a lower portion that is a calibrated capillary tube. These tubes have been employed by biologists to measure the PCV of algal suspension cultures (Sorokin, 1973). Depending on the density of the culture, it may be necessary to dilute or to concentrate the sample before placing it in the tube. For example, a relatively large sample (100 cm^3) can be given a precentrifugation to condense the cells in order for an appropriate reading to appear on the calibrated scale. Any dilutions or concentrations must be taken into consideration in the final calculation of the PCV.

2. Place the sample in the receiver of the centrifuge tube, and spin it at 2,000 × g for a period of 5 min. If this does not provide a clear demarcation of the cell boundary, then increase the time of centrifugation. The readings must be made immediately following the centrifugation because any delay will give inflated values owing to cell expansion. Several replications should be run simultaneously and a mean value determined. The results are expressed as cubic centimeters of cell pellet per cubic centimeter of culture.

Experiment 5. Determination of mitotic index (MI)

Because of the difficulties encountered in the maceration of callus, it is advisable that the student perform this experiment on a sample of actively dividing suspension culture. The following procedure has been adapted from Wetter and Constabel (1982).

1. The carbol-fuchsin technique requires the preparation of three stock solutions. Stock A consists of basic fuchsin (3 g) dissolved in 100 cm^3 ethanol (70% v/v), and this solution is completely stable. Stock B is prepared by adding 10 cm^3 of stock A to 90 cm^3 phenol (5% w/v). This reagent (B) should be discarded after 2 weeks. Stock C consists of 45 cm^3 of stock B, 6 cm^3 glacial acetic acid, and 6 cm^3 formaldehyde (37% v/v). The latter solution (C) was employed as a carbol-fuchsin stain by Carr and Walker (1961). The present experiment uses a modified carbol-fuchsin stain prepared by adding 2–10 cm^3 of stock C to 90–98 cm^3 acetic acid (45% v/v) and 1.8 g sorbitol. The optimum concentration of the stain, varying from 2 to 10%, depends on the plant species to be

stained. The staining reagent is more effective if it is prepared at least 2 weeks in advance and stored at room temperature (Kao, 1982).

2. Pipette a 5-cm^3 sample from the culture, and fix the cells in 1 cm^3 of a mixture of glacial acetic acid and ethanol (95% v/v) in a ratio of 1:3. Transfer a droplet of the fixed cells to a slide, and add an equal amount of the modified carbol-fuchsin stain. Wait about 5 min, add a cover slip to the absorbent paper, and squash the preparation by gently rolling a short glass rod over the cover slip. The red-stained nuclei will be visible in the microscope.

3. Examine microscopically a minimum of 500 nuclei, and categorize them as either (a) nondividing or (b) exhibiting some stage of mitosis (prophase–telophase). It is helpful if the student is provided with a set of photomicrographs showing plant cell mitosis so that the early and late stages will not be overlooked.

The mitotic index (MI) represents the percentage of total nuclei displaying mitosis at a given time in a sample:

$$\text{MI} = \frac{\text{number of nuclei in mitosis}}{\text{total number of nuclei examined in sample}} \times 100$$

Experiment 6. Tracheary element counts in primary explants

This experiment involves the maceration of primary explants and the associated callus following culture for varying periods of time on a xylogenic medium. The initial procedure should be followed as outlined in Chapter 7 on the induction of xylem differentiation.

1. Sacrifice the explants after 5, 10, and 15 days of incubation, and examine 10 explants for each time period. Place the explants individually in small vials, and immerse them in 1 cm^3 of a maceration reagent consisting of chromium trioxide and hydrochloric acid (Steward and Shantz, 1956). They should remain in the maceration reagent for 24 hr at room temperature (Brown and Rickless, 1949). Because of the toxicity of the fumes, the maceration reagent should be added to the vials by Pasteur pipette in the hood. Stopper the vials, and, if possible, store them in the hood.

2. After the maceration period, withdraw the acid mixture and replace it with approximately 0.5 cm^3 distilled water. Care must be exercised not to touch the fragile explants with the tip of the Pasteur pipette, and not to remove any tissue that has crumbled from the sample. Attach a no. 22-gauge needle to a syringe, and break up the sample with the tip

of the needle until the disrupted cells can be drawn into the syringe Expel the cells back into the vial. Repeat this pumping action until the sample has been thoroughly homogenized.

3. Transfer the macerated sample by syringe to a volumetric tube calibrated to 2 cm^3. Add distilled water until the level is precisely at the 2-cm^3 calibration mark. Add the cover slip diagonally across the Sedgwick-Rafter chamber so that spaces remain open at the opposite corners. *Note:* A suitable volumetric tube can be made from one of the small vials. Pipette 2 cm^3 of water into the vial. Mark the water level by attaching a strip of opaque plastic tape to the bottom of the meniscus.

4. Attach a no. 18-gauge needle to the syringe, and thoroughly mix the sample by pumping it repeatedly in and out of the syringe. Deliver 1 cm^3 of the sample to the Sedgwick-Rafter chamber, and slide the cover slip into the proper position (Guillard, 1973). If available, a green filter will aid in the detection of the tracheary elements.

5. The counting technique and calculations are similar to the procedure outlined in experiment 3. The dilution factor (DF), however, must be taken into account in the present calculations. The final volume of the sample was 2 cm^3, and DF equals 2. The total number of tracheary elements in the sample (ΣTE) may be expressed as:

$$\Sigma TE = 537.6(N)(DF)$$

If the mean tracheary element count per Whipple field is 20, then the sample contains 21,504 tracheary elements. If the sample has extremely large numbers of tracheary elements, then it is necessary to dilute the sample to a final volume of 4 or possibly 8 cm^3. For greater accuracy in counting, the number of tracheary elements in a Whipple field should not exceed approximately 25–30.

QUESTIONS FOR DISCUSSION

1. What are some reasons why the fresh weight and dry weight of a callus sample might not be reliable measurements of the growth rate of the culture?
2. In regard to quantitation, what are some characteristics that algal cultures and higher plant cell cultures have in common?
3. In addition to the use of chromium trioxide, what are some other methods that have been used for separation of plant cells?
4. What are some sources of experimental error involved in the use of the Sedgwick-Rafter chamber?
5. What information about a given culture does the mitotic index give the investigator? Why is the MI not a reliable indicator of the degree of cell division synchrony of a culture?

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18

Special topics

Virus eradication

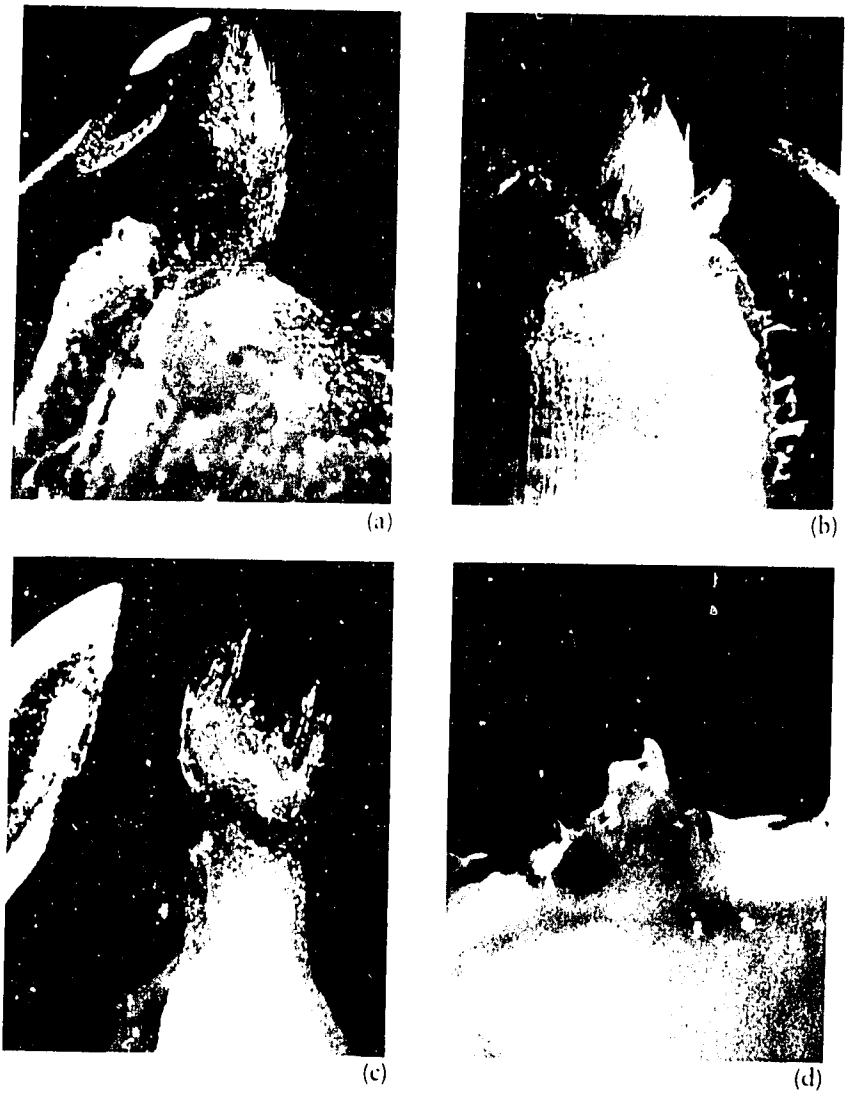
At a time when increasing population places pressure on agriculturists to increase the yield of foodstuffs, it is highly important that disease-free plant material be used. Virus infection can severely limit the yield of a wide range of crop plants (Over de Linden and Elliott, 1971). If vegetatively propagated plants are once systemically infected with a viral disease, the pathogen readily passes from one generation to the next. The entire population of a clonal variety may become infected with the same pathogen (Wang and Hu, 1980). In addition to virus transmission by asexual plant propagation, viral diseases are also spread by infected seeds (Hu and Wang, 1983).

Some care must be exercised in the terminology we use. The term "virus-free" means that a given plant is no longer infected with any viruses that can be detected by one or more virus-indexing techniques. Unknown viruses, however, may still be present within the plant tissues. It is preferable to apply the term "virus-tested" to these plants, since they may not literally be virus free.

A large number of plants have been successfully freed from viral infection through tissue-culture virus-eradication programs (see Wang and Hu, 1980; Hu and Wang, 1983). In addition to disease elimination, tissue culture techniques are also important in the development of disease-resistant plants (Helgeson and Haberlach, 1980; Earle, 1982).

The distribution of virus particles within the infected plant is not uniform (Limasset and Cornuet, 1949), and root and shoot apices are often devoid of virus particles (White, 1934; Holmes, 1948). Morel and Martin (1952) first demonstrated the elimination of viruses from *Dahlia* by the use of apical meristem cultures. This uneven distribution of virus particles has been used as the basis for in vitro eradication. The smallest shoot meristem explants are used, normally only encompassing the apical

Fig. 18.1. Dissection of the apical meristem for virus eradication. The sequence a–d shows a progressive dissection of the shoot tip (a) until only the apical dome and two leaf primordia remain (d). (Reprinted with permission from N. Espinoza, R. Estrada, P. Tovar, J. Bryan, and J. Dodds, Tissue culture micropropagation, conservation, and export of potato germplasm. Specialized Technology Document 1. Lima: International Potato Center, 1984.)

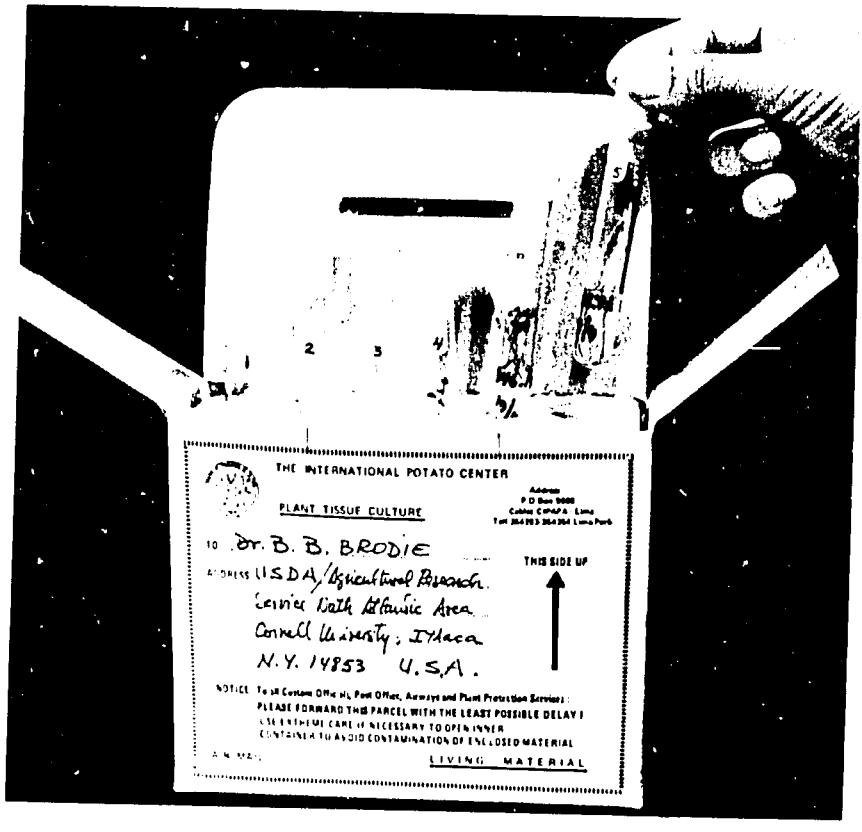


dome. A few leaf primordia may be included, because very minute ex plants are unlikely to produce roots. The size of the explant is critica to the success of the procedure, since only the uppermost tissue is com pletely free of virus. Figure 18.1 shows photographically the sequentia dissection of a meristem. According to Walkey (1980) most workers hav used shoot tips measuring between 0.5 and 1.0 mm in diameter.

Apical meristem culture may be coupled with a thermotherapy treat ment (Stace-Smith and Mellor, 1970). This technique involves growing the plants for 6–12 weeks at 30–40°C prior to the excision of the apica meristems (Walkey, 1980). An alternative approach is to culture the meristem explants at 30–40°C. At this elevated temperature viruses are heat inactivated, although the plant tissues will continue to grow.

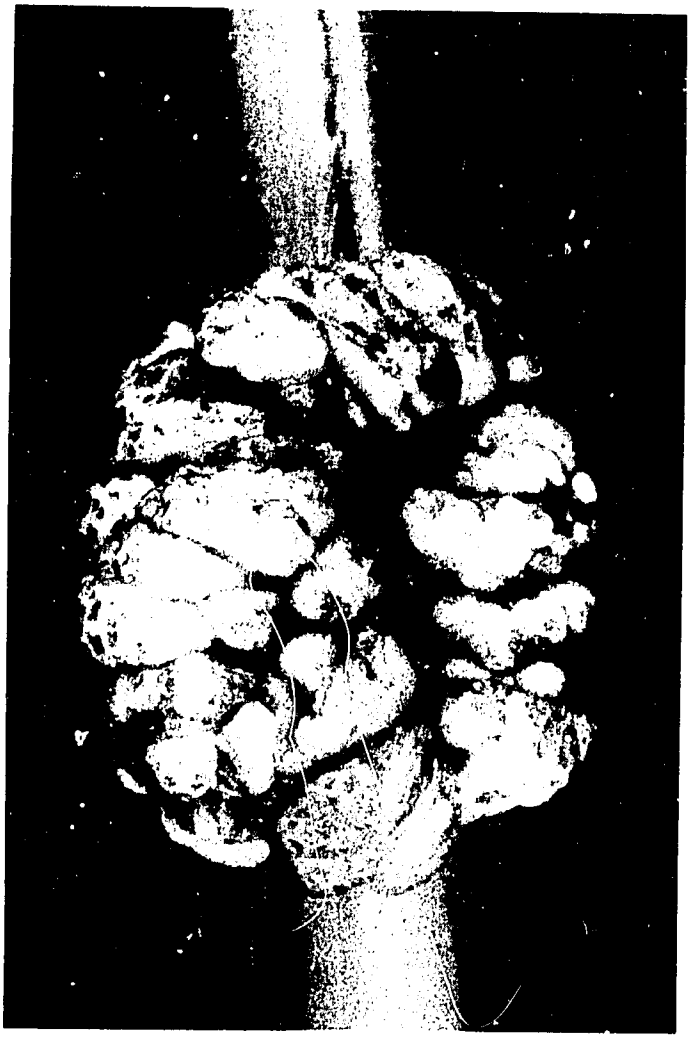
It should be emphasized that virus eradication by apical meristem cul- ture is not always successful, and consequently these cultures must be subjected to a rigorous testing program. Methods for testing for the

Fig. 18.2. Cultures of potato shoots packaged for international export.



presence of the virus in the cultures include infectivity assays, serological tests, and ultrastructural observations. Any material found to be infected is destroyed, and only proven "clean" cultures are permitted to enter a micropropagation program. It is necessary to rear only a single healthy uninfected plantlet from a diseased parent, and this individual can serve for micropropagation or multiplication by conventional means (Walkey, 1980).

Fig. 18.3. A tumor induced by infection with the crown gall bacterium (*Agrobacterium tumefaciens*) on sunflower (*Helianthus annuus*) stem. (Courtesy of D. Butcher.)

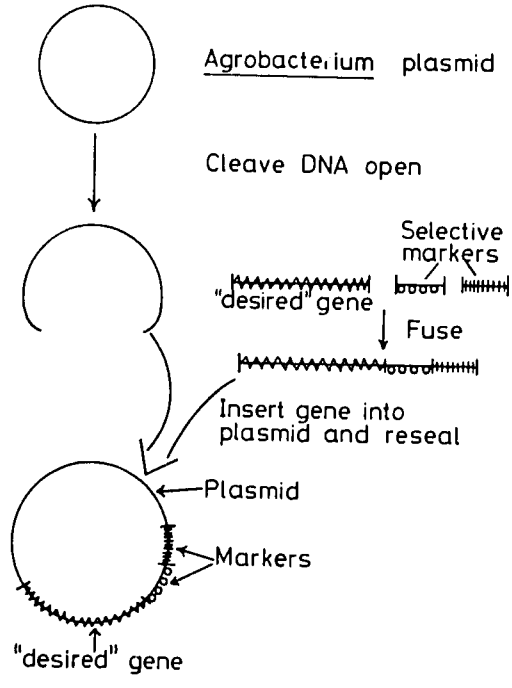


Strict quarantine regulations govern the international exchange of plant material. The use of in vitro pathogen elimination has greatly facilitated the international exchange of germplasm. Figure 18.2 shows a package of pathogen-tested potato shoot cultures. These cultures are ready for export to be used in the national potato program of a developing country.

Plant tumors and genetic engineering

The disorganized growth of the crown-gall tumor (Fig. 18.3) has received considerable attention from plant scientists since the early studies of Braun in 1942 (see Braun, 1974; 1975). The tumor is induced in susceptible plant material following infection of a wound with *Agrobacterium tumefaciens*. In the 1960s and 1970s many studies were undertaken to try to identify the "tumor-inducing principle." This unknown factor, discovered later to be a small ring of DNA or Ti plasmid, was found only in virulent strains of the bacterium. At the time of infection a segment of the Ti plasmid, termed T-DNA, is inserted into the genome or nuclear

Fig. 18.4. Diagramatic scheme outlining the basic principles of gene insertion into an isolated plasmid.



DNA of the host plant (Watson et al., 1975; Chilton et al., 1977). Genes contained within the T-DNA segment are operational within the host cell in the biosynthesis of unusual metabolites termed opines. These opines are metabolized by *Agrobacterium* for sources of carbon and nitrogen in the nutrition of the pathogen (Barton and Brill, 1983). The Ti plasmid may provide the genetic engineer a vector for the introduction of new genes into a host protoplast. Transformed protoplasts of tobacco containing T-DNA can regenerate a plant that is also transformed, i.e., contains operational foreign genes (Wullems et al., 1982). Although the biochemistry is complex, the concept is relatively simple (see Fig. 18.4). For example, a desired gene coding for a protein rich in essential amino acids is inserted into the Ti plasmid. In the same plasmid are inserted marker genes, for example, resistance to a particular antibiotic. The reconstructed Ti plasmid is now returned to the intact *Agrobacterium*, and one selects for "transformed" bacteria. Suitable plant material can then be infected with the transformed bacteria, and the new gene will be introduced into the higher plant genome.

During the past two years detailed studies have been made of the integration and expression of these new genes, which may be either natural or synthetic in origin (Hernalsteens et al., 1980; Ursic, Slightom, and Kemp, 1983; Herrera-Estrella et al., 1983a,b; in press; Schroder et al., 1984).

The use of plasmids as vectors to carry novel genes into plant cells opens a new era in plant genetics (Dodds, in press). Although the technology is still in its infancy, in the future it may be possible to increase photosynthetic efficiency, improve nutritional value of storage proteins, and increase resistance to environmental factors and plant predators. There is still, however, a long way to go before the results are commensurate with the ideas.

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FORMULATIONS OF TISSUE CULTURE MEDIA
(mg/l)

Ingredients	MS[1]	B5[2]	WH[3]	SH[4]
$(NH_4)NO_3$	1,650			
$(NH_4)_2SO_4$		134		
$(NH_4)H_2PO_4$				300
KNO_3	1,900	2,500	80	2,500
$Ca(NO_3)_2 \cdot 4H_2O$			288	
$CaCl_2 \cdot 2H_2O$	440	150		200
$MgSO_4 \cdot 7H_2O$	370	250	737	400
$Na_2SO_4 \cdot 10H_2O$			460	
KH_2PO_4	170			
$NaH_2PO_4 \cdot H_2O$		150	19	
KCl			65	
$FeSO_4 \cdot 7H_2O$	27.8	27.8	27.8[a]	15
Na_2EDTA	33.6[b]	33.6[b]	33.6[b]	20
$MnSO_4 \cdot 4H_2O$	22.3		6.65	
$MnSO_4 \cdot H_2O$		10		10
$ZnSO_4 \cdot 7H_2O$	8.6	2.0	2.67	1.0
H_3BO_3	6.2	3.0	1.5	5.0
KI	0.83	0.75	0.75	1.0
MoO_3			0.0001[c]	
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25		0.1
$CuSO_4 \cdot 5H_2O$	0.025	0.025	0.001[c]	0.2
$CoCl_2 \cdot 6H_2O$	0.025	0.025		0.1
myo-inositol	100	100		1,000
nicotinic acid	0.5	1.0	0.5	5.0
pyridoxine·HCl	0.5	1.0	0.1	0.5
thiamine·HCl	0.1	10.0	0.1	5.0
glycine	2.0		3.0	
sucrose	30,000	20,000	20,000	30,000
kinetin	0.40–10	0.1		
IAA	1–30			
2,4-D		0.1–1.0		0.5
p-chlorophenoxyacetic acid				2.0
pH	5.7	5.5	5.5	5.8

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[a] White's (1963) medium required 2.5 mg/l $Fe_2(SO_4)_3$. Since ferric sulfate precipitates easily, it is most convenient to use the MS formulation in the chelated form. Equivalent concentrations of iron provided by other reagents are given in Singh and Krikorian (1981).
[b] Revised from Murashige and Skoog's (1962) medium. See Singh, M., and Krikorian, A. D. (1980). Chelated iron in culture media. *Ann. Bot. 46*, 807–9.
[c] See White (1963), p. 59.

COMMERCIAL SOURCES OF SUPPLIES

Within the text reference is made to special chemicals, supplies, and equipment. The following is a brief list of the names and addresses of some suppliers. The inclusion of a particular supplier does not imply endorsement by the authors exclusive of other companies offering a similar product.

Agar and premixed tissue culture media

Carolina Biological Supply Company, 2700 York Road, Burlington, NC 27215, U.S.A.

Difco Laboratories, P.O. Box 1058A, Detroit, MI 48232, U.S.A.

Difco Laboratories Ltd., P.O. Box 14B, Central Avenue, East Molesey, Surrey KT8 OSE, U.K.

Flow Laboratories Inc., 7655 Old Springhouse Road, McLean, VA 22102, U.S.A.

Flow Laboratories Ltd., P.O. Box 17, Second Avenue, Industrial Estate, Irvine KA12 8NB, Ayrshire, Scotland, U.K.

FMC Corporation, Marine Colloids Division, BioProducts Department, 5 Maple Street, Rockland, ME 04841, U.S.A.

Gibco Laboratories, 3175 Staley Road, Grand Island, NY 14072, U.S.A.

Gibco-Biocult Ltd., P.O. Box 35, 3 Washington Road, Sandyford Industrial Estate, Paisley, Scotland PA3 3EP, U.K.

KC Biological Inc., P.O. Box 14848, Lenexa, KS 66215, U.S.A.

Marine Colloids (representative), Miles Scientific, Division of Miles Laboratories Ltd., Stoke Court, Stoke Poges, Slough, Berkshire SL2 4EB, U.K.

Oxoid U.S.A. Inc., 9017 Red Branch Road, Columbia, MD 21045, U.S.A.

Oxoid Ltd., Wade Road, Basingstoke, Hants, RG24 OPW, U.K.

Biochemicals

Aldrich Chemical Company, P.O. Box 355, Milwaukee, WI 53201, U.S.A.

Aldrich Chemical Company Ltd., The Old Brickyard, New Road, Gillingham, Dorset SP8 4JL, U.K.

Calbiochem-Behring, Division of American Hoechst Corporation, P.O. Box 12087, San Diego, CA 92112, U.S.A.

C P Laboratories Ltd., P.O. Box 22, Bishops Stortford, Herts CM22 7RQ, U.K.

Gallard-Schlesinger Chemical Mfg. Corporation, 584 Mineola Avenue, Carle Place, NY 11514, U.S.A.

ICN K&K Laboratories, 121 Express Street, Plainview, NY 11803, U.S.A.
Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178, U.S.A.
Sigma (London) Chemical Company Ltd., Fancy Road, Poole, Dorset BH17 7NH, U.K.
United States Biochemical Corporation, P.O. Box 22400, Cleveland, OH 44122, U.S.A.

Cellulose cell wall stain

Polysciences, Inc., Paul Valley Industrial Park, Warrington, PA 18976, U.S.A. (Calcofluor White M2R, purified; "Cellufluor")

Enzyme preparations

Calbiochem-Behring Corporation, P.O. Box 12087, San Diego, CA 92112, U.S.A. (Cellulysin; Macerase)
C P Laboratories Ltd., P.O. Box 22, Bishops Stortford, Herts CM22 7RQ, U.K. (representative Calbiochem-Behring; Cellulysin; Macerase)
Meiji Seika Kaisha Ltd., International Division, 4-16 Kyobashi, 2 chome, Chuo-ku, Tokyo, 104 Japan (Meicelase CESB, Meicelase CMB)
Plenum Scientific Research Inc., 210 Lee Place, Hackensack, NJ 07601, U.S.A. (Driselase)
Rohm & Haas Company, Independence Mall West, Philadelphia, PA 19105, U.S.A. (Rhozyme HP-150 Concentrate)
Seishin Pharmaceutical Company Ltd., Tokyo Branch, 4-13 Koamicho Nihonbashi, Tokyo, Japan (Pectolyase Y-23)
Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178, U.S.A. (Cellulysin, Driselase, hemicellulase, Macerase, pectinase, Pectolyase)
Sigma (London) Chemical Company Ltd., Fancy Road, Poole, Dorset BH17 7NH, U.K. (same enzyme preparations as in preceding entry)
R. W. Unwin & Company Ltd., Prospect Place, Welwyn, Herts AL6 9EW, U.K. (Cellulase Onozuka R10, Cellulase Onozuka RS, Meicelase, Macerozyme R-10, Pectolyase Y-23)
Yakult Honsha Co. Ltd., Nishinomiya Office, 8–21 Shingikancho, Nishinomiya 662, Japan (Cellulase Onozuka R-10, Cellulase Onozuka RS, Macerozyme R-10)

Filtration equipment

Gelman Sciences, 600 S. Wagner Road, Ann Arbor, MI 48106, U.S.A.
Henry Simon Ltd., P.O. Box 31, Stockport, Cheshire, SK3 ORT U.K. (nylon screening cloth)
Micron Separations Inc., 58 North Main Street, Honeoye Falls, NY 14472, U.S.A.
Millipore/Continental Water Systems, Ashby Road, Bedord, MA 01730, U.S.A.

Millipore (U.K.) Ltd., 11–15 Peterborough Road, Harrow, Middlesex HA 2YH, U.K.
Nuclepore Corporation, 7035 Commerce Circle, Pleasanton, CA 94566, U.K
Sartorius Filters Inc., 26575 Corporate Avenue, Hayward, CA 94545, U.S.A.
Sartorius-Membranfilter GmbH, P.O. Box 142, 34 Göttingen, FRG
Schleicher & Schuell Inc., 543 Washington Street, Keene, NH 03431, U.S.A.
Schleicher & Schüll AG, CH-8714 Feldbach ZH, Switzerland
Tetko Inc., 420 Saw Mill River Road, Elmsford, NY 10523, U.S.A.
Whatman Laboratory Products Inc., Whatman Paper Division, 9 Bridewell Place Clifton, NJ 07014, U.S.A.
Whatman Ltd., Springfield Mill, Maidstone, Kent ME14 2LE, U.K.

Glassware and plastics

Becton Dickinson U.K. Ltd., 21 Between Towns Road Cowley, Oxford, OX4 3LY, U.K.
Bellco Glass Inc., P.O. Box B, 340 Edrudo Road, Vineland, NJ 08360, U.S.A.
Cole-Parmer Instrument Company, 7425 North Oak Park Avenue, Chicago, IL 60648, U.S.A.
Corning Glass Works, Science Products, Corning, NY 14831, U.S.A. (Pyrex)
Costar, 205 Broadway, Cambridge, MA 02139, U.S.A.
Falcon, Division of Becton, Dickinson & Company, Box 243, Cockeysville, MD 21030, U.S.A.
Kernick & Son, Scientific Centre, Pentwyn, Cardiff CF2 7XF, U.K.
Kimble, Division of Owens-Illinois, P.O. Box 1035, Toledo, OH 43666, U.S.A.
Magenta Corporation, 4149 West Montrose Avenue, Chicago, IL 60641, U.S.A.
Nalgene Labware Department, Nalge Company, P.O. Box 365, Rochester, NY 14602, U.S.A.
Sterilin Products, 43–45 Broad Street, Teddington, TW11 8QZ, U.K.
Vangard International, 1111A Green Grove Road, Neptune, NJ 07753, U.S.A. (Nunc Products)
Wheaton Scientific, 1000 North Tenth Street, Millville, NJ 08332, U.S.A.

Glassware cleaning agents

International Products Corporation, P.O. Box 118, Trenton, NJ 08601, U.S.A. (Micro)

Laminar flow cabinets and microbiological hoods

The Baker Company Inc., Sanford Airport, Sanford, ME 04073, U.S.A.
Bellco Glass Inc., 340 Edrudo Road, Vineland, NJ 08360, U.S.A.
Carolina Biological Supply Company, 2700 York Road, Burlington, NC 27215, U.S.A.
Clean Room Products, 56 Penataquit Avenue, Bay Shore, NY 11710, U.S.A.

A. Gallenkamp & Company Ltd., P.O. Box 290, Technico House, Christopher Street, London EC2P 2ER, U.K.
Germfree Laboratories, 7435 NW 41 Street, Miami, FL 33166, U.S.A.
Hepair Mfg Ltd., Station Road, Thatcham, Berkshire, RG13 3JE, U.K.
Labconco Corporation, 8811 Prospect, Kansas City, MO 64132, U.S.A.

Magnifiers with built-in illuminators

Manning Holoff Company, 14603 Arminta Street, Van Nuys, CA 91402 (Magna-Lite)

Microscopes

American Optical Scientific Instruments, Box 123, Buffalo, NY 14240, U.S.A.
Bausch & Lomb, Box 743, Rochester, NY 14603, U.S.A.
E. Leitz Inc., 24 Link Drive, Rockleigh, NJ 07647, U.S.A.
E. Leitz (Instruments) Ltd., 30 Mortimer Street, London W1N 8BB, U.K.
Nikon, 623 Stewart Avenue, Garden City, NY 11530, U.S.A.
Olympus Corporation of America, Scientific Instrument Division, 4 Nevada Drive, New Hyde Park, NY 11042, U.S.A.
Vickers Instruments, Haxby Road, York YO3 75D, U.K.
Carl Zeiss (Oberkochen) Ltd., Degenhardt House, 30–36 Foley Street, GB-London, W1P 9AP, U.K.
Carl Zeiss Inc., One Zeiss Drive, Thornwood, NY 10594, U.S.A.

Polyester fleece

Pellon Corporation, Industrial Products Division, 491 Dutton Street, Lowell, MA 01852, U.S.A.

Polypropylene film

Amoco Corporation, Naperville, IL 60566 (specify type A 300)
Polypropylene Films Corporation, 1571 Timber Court, Elgin, IL 60120 (specify type 07 or 08, uncoated, 1 mil in thickness)

Seeds

Burpee Seed Company, Warminster, PA 18991; Clinton, IA 52732; or Riverside, CA 92502
Earl May Seed and Nursery Company, Shenandoah, IA 51603
Gurney Seed Company, Yankton, SD 57078
Nichols Garden Nurseries, 1190 North Pacific Highway, Albany, OR 97321
Thompson & Morgan Inc., P.O. Box 100, Farmingdale, NJ 07727

Shakers and roller culture apparatus

Baird & Tatlock (London) Ltd., P.O. Box 1, Romford, Essex RM1 1HA, U.K.
Bellco Glass Inc., 340 Edrudo Road, Vineland, NJ 08360, U.S.A.
A. Gallenkamp & Company Ltd., P.O. Box 290, Technico House, Christopher Street, London EC2P 2ER, U.K.
Lab-Line Instruments, 15 S Bloomingdale Avenue, Melrose Park, IL 60160, U.S.A.
New Brunswick Scientific Company Inc., 44 Talmadge Road, Edison, NJ 08817, U.S.A.
Wheaton Scientific, 1000 North Tenth Street, Millville, NJ 08332, U.S.A.

Surgical and culture instruments

Carolina Biological Supply Company, 2700 York Road, Burlington, NC 27215, U.S.A.
Clay Adams, 299 Webro Road, Parsippany, NJ 07054, U.S.A.
Techi-Tool, 5 Apollo Road, Plymouth Meeting, PA 19462, U.S.A.

Tissue culture apparatus

Baird & Tatlock (London) Ltd., P.O. Box 1, Romford, Essex RM1 1HA, U.K.
Bellco Glass Inc., 340 Edrudo Road, Vineland, NJ 08360, U.S.A.
Carolina Biological Supply Company, 2700 York Road, Burlington, NC 27215, U.S.A.
Corning Glass works, Science Products, Corning, NY 14831, U.S.A.
Costar, 205 Broadway, Cambridge, MA 02139, U.S.A.
A. Gallenkamp & Company Ltd., P.O. Box 290 Technico House, Christopher Street, London EC2P 2ER, U.K.
Germfree Laboratories, 7435 NW 41 Street, Miami, FL 33166, U.S.A.
New Brunswick Scientific Company Inc., 44 Talmadge Road, Edison, NJ 08817, U.S.A.
Wheaton Scientific, 1000 North Tenth Street, Millville, NJ 08332, U.S.A.

The American Type Culture Collection has established a plant tissue culture bank. Currently 62 well-documented cultures are maintained under cryopreservation conditions and are available to the scientific community for a nominal fee. For additional information contact the Professional Services Department, American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852 USA.

AUTHOR INDEX

Pages from the sections of Selected References are in italic.

SUBJECT INDEX