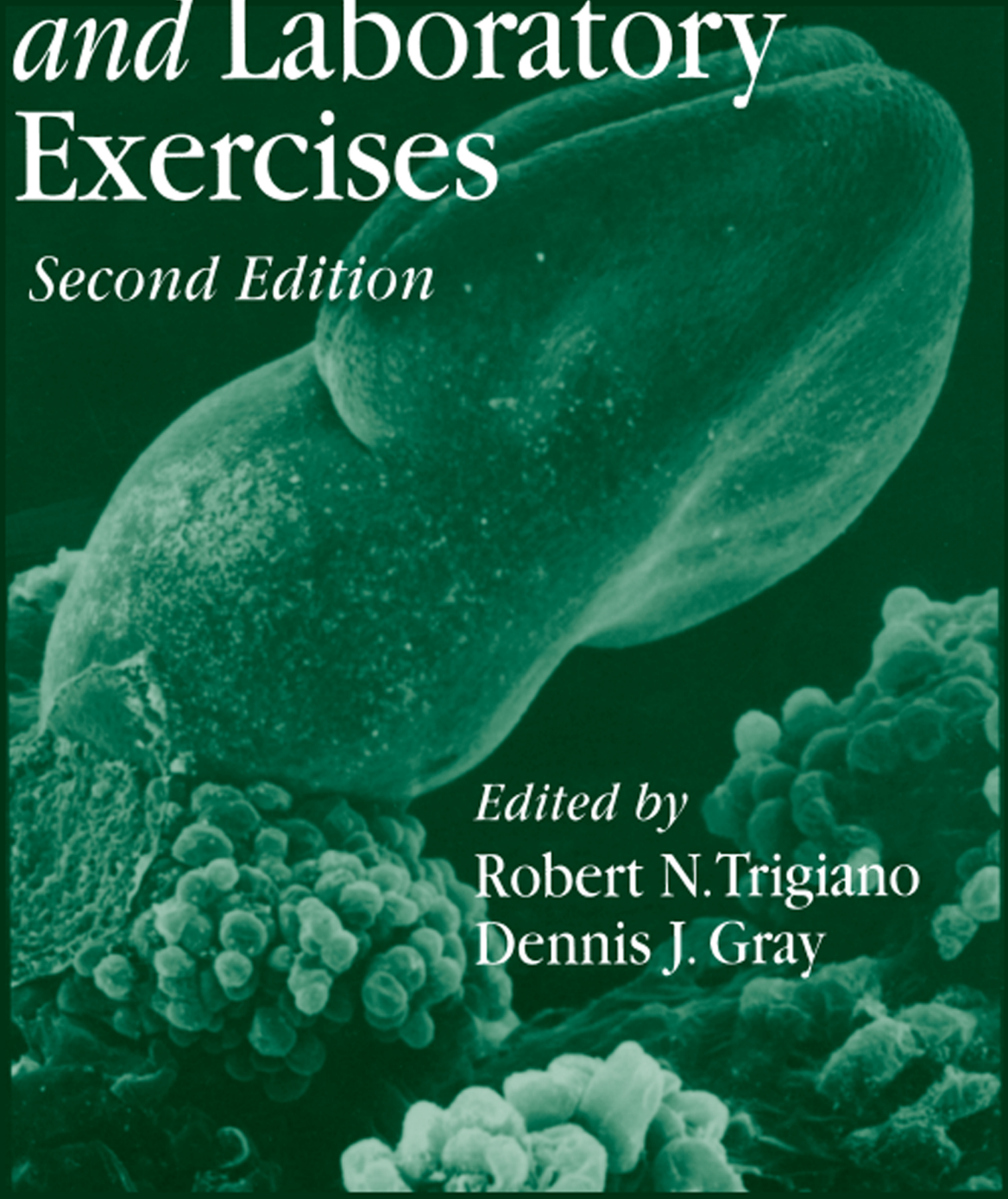
Plant Tissue Culture Concepts *and* Laboratory Exercises

Second Edition



Edited by

Robert N. Trigiano

Dennis J. Gray

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Cover Photograph: Somatic embryo of grape
(see Chapter twenty-one). Courtesy of D. J. Gray.

Library of Congress Cataloging-in-Publication Data

Plant tissue culture concepts and laboratory exercises / edited by
Robert N. Trigiano, Dennis J. Gray — 2nd ed.
p. cm.
Includes bibliographical references and index.
ISBN 0-8493-2029-1 (alk. paper)
1. Plant tissue culture Laboratory manuals. I. Trigiano, R. N.
(Robert Nicholas), 1953- . II. Gray, Dennis, J. (Dennis John),
1953-
QK725.P584 1999
571.5'.382—dc21 99-34987
CIP

Direct all inquiries to CRC Press LLC, 2000 N.W. Corporate Blvd., Boca Raton, Florida 33431.

Visit the CRC Press Web site at www.crcpress.com

International Standard Book Number 0-8493-2029-1
Library of Congress Card Number 99-34987
Printed in the United States of America 5 6 7 8 9 0
Printed on acid-free paper

Overview and scheduling of laboratory exercises

The book is arranged such that the "typical" or "common" supplies and media formulations for all of the laboratory exercises are listed in Chapter three, "Getting started with tissue culture — media preparation, sterile technique, and laboratory equipment." However, unique supplies and media are given in each chapter when necessary. In particular, student supplies, such as scalpels and blades, are described in Chapter three under "Equipment and supplies for a tissue culture laboratory"; those items are not listed in individual chapters. We recommend that each student or team of students be provided with a kit containing those instruments and supplies. Reference to specific products or suppliers does not constitute endorsement or criticism of similar ones not mentioned.

Although arranged by topic, several laboratory exercises from different topic areas use the same plant species. Therefore, it might be most efficient to conduct laboratories on a range of topics concurrently in order to efficiently utilize certain plant material. To assist in planning, the plant species are referenced to laboratory chapters in Table 1.

We encourage instructors to take the time to peruse the laboratory exercises well in advance of the anticipated laboratory starting date. Some experiments will require starting stock plants. Other experiments will depend on cultures started or obtained from outside sources weeks or months ahead of time. For example, most of the genetic transformation and in vitro plant pathology exercises require plant pathogenic bacteria, e.g., *Agrobacterium tumefaciens* and/or *Pseudomonas syringae*. Note that regardless of the source of the pathogens (the American Type Culture Collection, a colleague at your institution, etc.), be sure to acquire the proper documentation and/or permits for the cultures. Preliminary planning will save time and disappointment later on and will make for a better experience for students and instructors.

Robert N. Trigiano

Dennis J. Gray

Table 1 Correlation of Plants to Laboratory Exercises for Scheduling Purposes

Plant	Chapter
Ajuga	38
Cabbage	34
Carnation	36
Carrot	32
Chrysanthemum	15, 28, 33
Cineraria	23
Dieffenbachia	10
Fern	41
Lilac	12
Melon	21, 34
Mint	36
Orchardgrass	20, 28
Peanut	22
Petunia	18
Spruce	25
Potato	11, 27, 36
Rose	13
Soybean	39
Syngonium	9
Tobacco	4, 27, 30, 32, 39
Torenia	17
Watermelon	16
Yellow poplar	24

Editors

Robert N. Trigiano, Ph.D., is Professor of Ornamental Plant Biotechnology in the Institute of Agriculture, Department of Ornamental Horticulture and Landscape Design at the University of Tennessee at Knoxville.

Dr. Trigiano received his B.S. degree with an emphasis in Biology and Chemistry from Juniata College, Huntingdon, PA in 1975 and an M.S. in Biology (Mycology) from the Pennsylvania State University in 1977. He was an Associate Research Agronomist, mushroom culture and plant pathology, for Green Giant Co., Le Sueur, MI until 1979 and then a Mushroom Grower for Rol-Land Farms, Ltd., Blenheim, Ontario, Canada during 1979 and 1980. He completed a Ph.D. in Botany and Plant Pathology (co-majors) at North Carolina State University at Raleigh in 1983. After concluding postdoctoral work in the Plant and Soil Science Department at the University of Tennessee, he was an Assistant Professor in the Department of Ornamental Horticulture and Landscape Design at the same university in 1987, promoted to Associate Professor in 1991 and to Professor in 1997.

Dr. Trigiano is a member of the American Society for Horticultural Science and the Mycological Society of America, and the honorary societies of Gamma Sigma Delta, Sigma Xi, and Phi Kappa Phi. He has been an Associate Editor for the journals of the American Society of Horticultural Science and *Plant Cell, Tissue and Organ Culture* and Editor for *Plant Cell Reports.* He has received the T.J. Whatley Distinguished Young Scientist Award (University of Tennessee, Institute of Agriculture, 1991) and the Gamma Sigma Delta Research Award of Merit (University of Tennessee, 1991).

Dr. Trigiano has been the recipient of several research grants from the U.S. Department of Agriculture (USDA), the Horticultural Research Institute, and from private industries. He has published more than 100 research papers, book chapters, and popular press articles. He teaches undergraduate/graduate courses in Plant Tissue Culture, Plant Disease Fungi, DNA Analysis, Protein Gel Electrophoresis, and Plant Microtechnique. Current research interests include somatic embryogenesis and micropropagation of ornamental species, fungal physiology, and population analysis.

Dennis J. Gray, Ph.D., holds the University of Florida Research Foundation Professorship and is a member of the Institute of Food and Agricultural Sciences' Horticulture Department. He directs the plant biotechnology program at the Central Florida Research and Education Center where he also serves as the Assistant Center Director.

Dr. Gray graduated with a B.A. degree in Biology from California State College, Stanislaus, in 1976 and received an M.S. degree in Mycology, with a minor in Botany from Auburn University in 1979. He earned a Ph.D. degree in Botany, with a minor in Plant Pathology from North Carolina State University in 1982. After a postdoctoral fellowship at the University of Tennessee, he joined the faculty of the University of Florida in 1984, reaching the rank of Professor in 1993.

Dr. Gray has been a member of the American Association for the Advancement of Science, the American Institute of Biological Sciences, the American Society for Horticul-

tural Science, the Botanical Society of America, the Council for Agricultural Science and Technology, the Society for In Vitro Biology, the International Association for Plant Tissue Culture and Biotechnology, the International Horticultural Society, and Sigma Xi. He was Associate Editor, then Managing Editor, of the internationally recognized refereed journal *Plant Cell, Tissue and Organ Culture* from 1988 through 1994.

Dr. Gray has received research grants and support from the Binational Research and Development Fund, the Florida Department of Agriculture and Consumer Affairs, the Florida High Technology and Industry Council, the U.S. Department of Agriculture, and private industry. He has been an author or co-author of more than 200 publications and holds several patents. His current interests include the developmental biology of regenerative plant cells and the integration of contemporary and newly emerging technologies for crop improvement.

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Acknowledgments

We would like to thank the contributing authors for their efforts and patience throughout this lengthy endeavor; Joy E. Nevine (JEN) was indispensable in producing the excellent computer drawings; the University of Tennessee's Institute of Agriculture and the University of Florida's Institute for Food and Agricultural Sciences for providing financial support for this project; and Bob Lyons for helping to develop the format of the "procedure boxes." We thank our spouses, Kay Trigiano and Amy Gray, for assistance with proofreading, production, and, of course, their undying support. Finally, we are indebted to Andrew N. Trigiano and Bob D. Gray who provided constant inspiration and a welcomed source of diversions during the completion of the second edition.

Contents

Part VI: Special topics

Part VII: Appendix

Part VIII: Index

Part I

Introduction

chapter one

Introduction to plant tissue culture

Dennis J. Gray and Robert N. Trigiano

The purpose of this chapter is to provide an initial focus to *Plant Tissue Culture Concepts and Laboratory Exercises* and to begin the process of defining terms and ideas unique to this subject area. Keeping in mind that the mission of the entire book is to introduce, define, and provide training, we will use this "introduction" primarily to orient the reader on the book's structure and to highlight information that is discussed in depth in subsequent chapters. We have taken a "minimalist" approach to development of definitions and use of terminology for the simple reason that this serves, in part, the role of an introductory textbook; in our experience, the specific meaning of many terms varies depending on whom is asked and it is often impossible to arrive at a globally satisfactory definition. Rather than confuse the beginning student (and ourselves) with "verbal gymnastics," we chose to simplify terminology. This results in the emphasis being placed on assimilation of key concepts and allows the student to become aware of the richness and natural controversy that involves terminology of such an emerging field after having acquired preliminary background knowledge and confidence. An example of our minimal approach to terminology can be seen in the very title of the book — "... plant tissue culture ...." To be painstakingly exact, the title should have been "... plant cell, tissue, organ, and organ system culture...." However, we achieved the desired effect of announcing the subject of the book without being tedious or confusing. This same approach represents our goal throughout the text.

Meant to serve as a primary text for both introductory and advanced courses, *Plant Tissue Culture Concepts and Laboratory Exercises* furnishes instructors and students alike with a broad consideration of the field — providing historical perspectives, discussing state-of-the-art techniques and methodologies, and looking forward to future advances and applications. It also presents many useful protocols and procedures and, thus, should serve as a valuable reference. The book is intentionally written to be rather informal — it provides the reader with a minimum number of references but does not sacrifice essential information or accuracy. Broad-topic chapters are authored by specialists with considerable experience in the field and are supported by one or more laboratory exercises illustrating central concepts of the topic. Collectively, the laboratory exercises are exceptionally diverse in nature, providing something for everyone — from beginning to advanced students. Importantly, the authors have successfully completed the exercises many times, often with either tissue culture classes or in their own research laboratories. A unique feature of all the laboratory exercises is that the authors have provided in general terms what results should be expected from each of the experiments. At the end of each exercise, there are a series

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of questions designed to provoke individual thought and critical examination of the experiment and results. Our intentions are that instructors will not attempt to do all the experiments, but rather select one or two for each concept that serves the needs and interests of their particular class. Thus, exercises for different crop types, such as grasses, ornamentals, trees, and vegetables, are variously provided to allow tailoring of a class to the department or discipline in which it is taught. For an advanced class, different experiments may be assigned among resourceful students. More advanced experiments following the general or beginning class exercises are embedded within some of the laboratory chapters.

The laboratory projects are executed with many different agronomic and horticulturally important plants including chrysanthemum, orchardgrass, syngonium, ajuga, petunia, watermelon, peanut, and lilac, all of which are easy to grow, can be ordered from common sources for nominal fees, or can be purchased at the local market. This range of plants is substantially broader than that used in any previous text on the subject and should stimulate interest in students from many botanical and agricultural disciplines.

The various chapters necessarily assume that the student has a good understanding of botany and botanical terminology. As such, it is recommended that companion plant anatomy, genetics, morphology, and physiological textbooks be available as needed in order to provide access to basic botanical knowledge.

In addition to this introductory section, the textbook is divided into the following five primary parts: History of Plant Tissue Culture, Supporting Methodologies, Propagation Techniques, Crop Improvement Techniques, and Special Topics. Each section combines related facets of plant tissue culture and includes one to several concept chapters, usually with accompanying laboratory exercises.

Part II, "History of Plant Tissue Culture," is an abbreviated account of how the field developed from an early theoretical base to the highly technical discipline that exists today. This treatment is somewhat unique because it documents research progress as modulated by significant world events. In addition to recording the people, places, and dates for pertinent discoveries, we feel it is interesting for students to see the challenges encountered by researchers that result in the often uneven pace of research. We end this chapter on a contemporary "hot" topic by discussing progress being made with genetically engineered plants in light of society's reaction, much of which information is gleaned from news reports. Beyond a past historical account, we hope that this chapter will provide a "snapshot" of the controversial present.

Part III, "Supporting Methodologies," begins the process of teaching tissue culture methodology. Chapter three covers several key topics that must be assimilated to accomplish subsequent laboratory exercises. It describes basic equipment needed and discusses the nature of nutrient culture medium and provides formulas for the most commonly used types. Chapter three also discusses various methods to prepare medium, and provides complete and logical examples of how to make solutions and dilutions and to accomplish sterile culture work. An experienced teacher with laboratory resources and methodologies already in place may choose to pay less attention to this chapter, whereas the instructor of a newly established course will find it indispensable. Chapter four is meant to bring the student into first contact with actual "tissue culture" by demonstrating the essential need for specific nutrients to grow "callus cultures." "Callus" is probably the most common term used to describe tissue cultures, but it only pertains to a certain type of unorganized tissue. As previously mentioned, a common term like "callus" can be defined in several, often conflicting, ways depending on opinion, but for here we consider a callus to be an unorganized tumor-like growth of cells. Part III ends with three chapters designed to emphasize common methods to visualize and document studies (histology and microscopy/photography, respectively) and to quantify responses (statistical analysis) of tissue culture in research.

Part IV, "Propagation Techniques," encompasses the essential foundation of plant tissue culture. In this section the three types of commonly used culture regeneration systems are introduced, then discussed and illustrated in depth.

We begin Part IV by discussing "Propagation from Preexisting Meristems" (Chapter eight), a process that is more commonly termed "micropropagation." Students tend to particularly enjoy some of the exercises in this section because they culminate in "house plants" that can be taken home and reared. Micropropagation is the simplest and most commercially useful tissue culture method. The tissue culture industry uses micropropagation almost exclusively for ornamental plant production. This is reflected by the range of ornamental plants used in the laboratory exercises. However, a unique exception to micropropagation of ornamental plants is the exercise on tissue culture production of potato (Chapter eleven); tissue culture-derived microtubers now are commonly used to establish potato fields. Other points of interest in this section are given in Chapter ten, which demonstrates the exact methodology used by industry to produce Dieffenbachia, and Chapter thirteen, which shows the production of rose flowers in culture.

The second propagation system to be discussed in Part IV is "Organogenesis" (Chapter fourteen). Organogenesis is the development of organs, typically shoots and/or roots, from cells and tissues that would not normally form them. The term "adventitious" has also been used to describe the plant parts formed by the process of organogenesis. Shoot organogenesis is another means of propagating plants. While not used much in commercial production, organogenesis is used extensively in genetic engineering as a means to produce plants from genetically altered cells. In Chapter fourteen, both the theory and developmental sequences of how cells are induced to follow such a developmental pathway are discussed. This chapter is followed by laboratory exercises that show the induction of shoots from leaves of chrysanthemum (Chapter fifteen), watermelon (Chapter sixteen), *Torenia* (Chapter seventeen), and petunia (Chapter eighteen).

The third propagation system discussed in Part IV is "Nonzygotic Embryogenesis" (Chapter nineteen). Nonzygotic embryogenesis is a broadly defined term meant to cover all instances where embryogenesis occurs outside of the normal developmental pathway found in the seed. One type of nonzygotic embryogenesis is termed somatic embryogenesis. This is a unique phenomenon exhibited by vascular plants, in which somatic (nonsexual) cells are induced to behave like zygotes. Such induced cells begin a complex, genetically programmed series of divisions and eventual differentiation to form an embryo that is more or less identical to a zygotic embryo. This type of propagation system is important since the embryos develop from single cells, which can be genetically engineered, and are complete individuals that are capable of germinating directly into plants. Thus, potentially, somatic embryogenesis also represents an efficient propagation system. Chapter nineteen discusses the developmental processes and significance of nonzygotic embryogenesis. Laboratory exercises illustrate nonzygotic embryogenesis for a range of crop types, including a grass (orchardgrass) (Chapter twenty), a vegetable (cantaloupe) (Chapter twenty-one), an agronomic crop (peanut) (Chapter twenty-two), a flowering ornamental plant (cineraria) (Chapter twenty-three), and both angiosperm (yellow poplar) and gymnosperm (white spruce) tree species (Chapters twenty-four and twenty-five, respectively).

In Part V, "Crop Improvement Techniques," the aforementioned propagation techniques are integrated with other methodologies in order to modify and manipulate germplasm. Chapter twenty-six discusses the use of plant protoplasts. Protoplasts are plant cells from which the cell wall has been enzymatically removed, making the cells amenable to cell fusion and other methods of germplasm manipulation. Two laboratory exercises concerning tobacco and potato (Chapter twenty-seven) and chrysanthemum and orchardgrass (Chapter twenty-eight) follow. Chapter twenty-nine details the use of

haploid culture in plant improvement. Haploid cultures usually are derived from microspore mother cells and result in cells, tissues, and plants with half the normal somatic cell chromosome number. Such plants are of great use in genetic studies and breeding, since all of the recessive genes are expressed and by doubling the haploid plants back to the diploid ploidy level, dihaploid plants are produced, which are completely homozygous. True homozygous plants are time consuming and often impossible to produce by conventional breeding. Chapter thirty is a laboratory exercise detailing haploid plant production from the microspores contained in tobacco anthers.

Concept Chapter thirty-one discusses genetic transformation (also known as genetic engineering), which is a current hot topic in agriculture. Transformation wherein genes from unrelated organisms can be integrated into plants without sexual reproduction, resulting in "transgenic plants," is the most significant application for plant tissue culture when considering its impact on humankind (see Part II). Two laboratory exercises discuss transformation of tobacco and carrot (Chapter thirty-two) and chrysanthemum (Chapter thirty-three) using *Agrobacterium tumefaciens,* nature's own and the original genetic engineer. This soil-inhabiting bacterium is ubiquitous and strains infect a wide range of host plants including angiosperms (monocots and dicots), gymnosperms, and ferns. *Agrobacterium* causes a tumor-like proliferation of cells, hence the common name of the disease is "crown gall," by transferring some of its plasmid (T_i) DNA to the host cell. Researchers have cleverly learned how to disarm (can no longer cause disease) *Agrobacterium* by deleting the genes that cause tumor growth. They can substitute genes that we want to transfer to our host species. Among these useful genes are those for disease resistance, herbicide tolerance, flower color, and fruit ripening. Chapter thirty-four describes the construction of a device for particle bombardment of plants, an alternate method of transformation. In this method, DNA is coated onto small tungsten or gold particles and literally shot into the plant cells.

Chapter thirty-five describes the use of cryopreservation of germplasm and Chapter thirty-six is a laboratory exercise illustrating its use. Cryopreservation is an efficient means of safeguarding valuable plant germplasm by freezing all metabolic activity. Cryopreserved cells and tissue can be kept for extended periods of time without mutations occurring or any physiological decline. Concept Chapter thirty-seven describes the production of secondary products by plant cells in culture. This subject is somewhat unique in the context of previous topics, because the end product is not a regenerated plant, but rather a chemical. Use of plants as biofactories to produce complex pharmaceuticals is of great interest, particularly due to its potential application in health care. In Chapter thirty-eight, the methods for production of an intensely colored pigment from *Ajuga* cell cultures are described. A final topic Part V is that of in vitro plant pathology. This topic (Chapter thirty-nine) is introduced to demonstrate the use of in vitro systems to mimic whole plants in the development of disease symptoms — overall, a convenient means of studying host-pathogen interactions.

Part VI, "Special Topics," is a bit of a "catch-all" section where we placed topics that we considered important enough to warrant inclusion in the book, but which did not quite fit in the other sections. Chapter forty discusses reasons that genetic and/or phenotypic variation occurs as a result of culture and presents up-to-date supporting research findings. Tissue culture of ferns (Chapter forty-one) illustrates the complete fern life cycle in a culture vessel. Beyond a prelude to propagation, it demonstrates alternation of generations, a basic biological principle. Chapter forty-two is a look into the mechanics of commercial plant production, which is the primary method of producing many house plants and is an important industry worldwide. This chapter validates the commercial application of many exercises presented in Part IV, "Propagation from Preexisting Meristems." Chapter forty-three describes the importance of clean cultures and problems

associated with maintaining in vitro cultures and Chapter forty-four is a companion laboratory exercise showing how plant pathogens are detected in cultured plants.

In addition to the information included in this book, explosive expansion of internet access in the last few years has provided new and almost indispensable learning resources for the student. We mention two useful listservers here and encourage students to log onto them for background information and additional support. One is the Plant-TC listserv, which was conceived and maintained by Dr. Mark Galatowitsch at the University of Minnesota. The Plant-TC listserv is an international forum for discussing scientific, educational, hobby, and other aspects related to plant tissue culture. To subscribe, send an e-mail message to "listserv@tc.umn.edu". Leave the subject line blank and enter the following as a message: "sub plant-tc YOUR NAME". Subscription is then automatic. Dr. Galatowitsch also maintains a list of plant tissue culture-related links at "http://www.agro.agri.unm.edu/planttc/optc.htm". Another internet service is Agnet, which is maintained by Dr. Douglas Powell at the University of Guelph. Agnet provides current news that focuses on agriculture and, particularly, the development and impact of transgenic crops worldwide. To subscribe to Agnet, send mail to: "listserv@listserv.uoguelph.ca". Leave the subject line blank and in the body of the message enter the following: "subscribe agnet-L YOUR NAME".

Based on the many telephone calls, letters, and e-mails that we received, the first edition of *Plant Tissue Culture Concepts and Laboratory Exercises* successfully facilitated training of students in current principles and methodologies of our rapidly evolving field. We asked for constructive criticisms from users of the book and employed them to make a number of improvements in this second edition. The most notable improvement is the addition of listed steps as procedure boxes in the laboratory exercises, which will make procedures easier to follow. Several chapters have been added, including this introduction, which was sorely lacking in the first edition. We believe that these improvements will ensure that the second edition will be even better than the first and, hopefully, will enjoy at least the same level of success! As always, we welcome comments from colleagues and students as they put the textbook to use.

Part II

History of plant tissue culture

chapter two

History of plant tissue and cell culture

James D. Caponetti, Dennis J. Gray, and Robert N. Trigiano

The field of plant tissue culture is based on the premise that plants can be separated into their component parts (organs, tissues, or cells), which can be manipulated in vitro and then grown back to complete plants. This idea of handling higher plants with the ease and convenience of microorganisms has conjured up many wonderful possibilities for their study and use; these possibilities, in turn, have been the long-standing stimuli driving research and development in the field. Plant tissue culture along with molecular genetics is a core technology for genetic engineering. After many years of overly optimistic promise, genetically engineered plants have finally reached the marketplace. At the end of this chapter, we discuss how the greatly anticipated societal benefits of this technology have been modulated by a good deal of concern regarding its safety.

The first successful plant tissue and cell culture was accomplished by Gottlieb Haberlandt near the turn of the 20th century when he reported the culture of leaf mesophyll tissue and hair cells (see Steward, 1968; Krikorian and Berquam, 1969). This was a remarkable accomplishment considering that little was known about plant physiology at the time. In retrospect, however, Haberlandt must have drawn on a body of previous knowledge in plant biology. We must assume that he was familiar with the writings of early philosophers such as Aristotle, Theophrastus, Pliny the Elder, Dioscorides, Avicenna, Magnus, Angelicus, and Goethe and that his studies surely must have included the anatomical observations of Hooke, Malpighi, Grew, Nageli, and Hanstein. His own research must have led him to the investigations by early plant physiologists such as van Helmont, Mariotte, Hales, Priestly, Ingenhousz, and Senebier and he must have had access to the morphological and physiological investigations of 19th century botanical researchers such as Schleiden, Schimper, Pringsheim, Unger, Hedwig, Hofmeister, Vochting, Sachs, Goebel, Bower, and Farlow. The information available from these sources coupled with improved light microscopes must have given Haberlandt the insight to culture plant cells and to predict that they could not only grow, but divide and develop into embryos and thence to whole plants, a scenario referred to as totipotency by Steward (1968).

Unfortunately, the cells that Haberlandt cultured did not divide and, thus, his ideas of plant development and totipotency did not come to fruition. The probable reason for the failure was that the plant growth regulators (PGRs) needed for cell division, proliferation, and embryo induction were not present in the culture medium. Indeed, PGRs had not yet been discovered. Apparently, Haberlandt became discouraged and pursued other

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physiological investigations. However, his ideas of embryo induction in culture did not go unnoticed in the scientific world. For example, Hannig (1904) cultured nearly mature embryos excised from seeds of several species of crucifers. Moreover, Haberlandt's lack of success was not in vain because one of his students, Kotte (1922), reported the growth of isolated root tips on a medium consisting primarily of inorganic salts. At the same time, and quite independently, Robbins (1922) reported a similar success with root tips and stem tips, and White (1934) reported that not only could cultured tomato root tips grow, but they could be repeatedly subcultured to fresh medium of inorganic salts supplemented with yeast extract, unbeknown to him a good source of B vitamins.

Innovative plant tissue culture techniques progressed rapidly during the 1930s due to the discovery that B vitamins and natural auxin were necessary for the growth of isolated tissues containing meristems. The growth-promoting effects of thiamine on isolated tomato root tips was reported by White (1937). A series of ingenious experiments with oat seedlings by Fritz Went in the 1920s plus other plant physiologists, including Kenneth Thimann, led to the discovery of the first PGR, indoleacetic acid (IAA). IAA is a naturally occurring member of a class of PGRs termed "auxins." The events leading to the discovery of IAA are well-documented in a report published in 1937. Duhamet (1939) reported the stimulation of growth of excised roots by IAA.

The avenue was now open for rapid progress in the successful culture of plant tissues during the 1930s. The culture of meristems other than root and shoot tips was explored. With improved culture media, La Rue (1936) achieved better success at culturing plant embryos compared to the efforts of Hannig 32 years earlier. Gautheret (1934) reported the successful culture of the cambium of several species of trees to produce callus on medium containing B vitamins and IAA. Further research with other meristematic tissues led Nobecourt (1937) and Gautheret (1939) to obtain callus from carrot root cambium and White (1939) to obtain tobacco tumor (crown gall) tissue. The rapid progress of plant tissue culture, however, was soon abruptly curtailed by the start of World War II in 1939.

Since one theater of World War II was concentrated in Europe, further progress in plant tissue culture by the European originators was almost impossible in the midst of the disruption and destruction and the shortages of laboratory supplies and equipment. Scientists in other locations also suffered shortages of laboratory supplies, equipment, and personnel. During the war years (1939 to 1945), nevertheless, some progress was made in plant tissue culture. Johannes Van Overbeek and associates reported they were able to obtain seedlings from heart-shaped embryos by enriching culture media with coconut milk besides the usual salts, vitamins, and other nutrients. Also by this time, Panchanan Maheshwari, along with associates and students in India, were very active in angiosperm embryology research that began before the war and progressed into the 1940s as described in his book (1950). Armin Braun reported on tumor induction related to crown gall disease. Experiments on tobacco tissues by Folke Skoog demonstrated organ formation in cultured tissues and organs. In Western Europe, some progress occurred in that Guy Camus was the first to report grafting experiments in tissue cultures and Georges Morel reported on developing techniques to culture parasitized plant tissues.

With war's end in the spring of 1945, a resumption of prewar tissue culture activity could not occur immediately. But some progress in plant tissue culture technology occurred. Ernest Ball reported on greatly improving the potential for culturing shoot tips, beginning with those of nasturtium and lupine. Albert Hildebrandt and co-workers made improvements on the medium for the growth of tobacco and sunflower tissues and Morel was well into applying tissue culture techniques to the study of parasites associated with plant tissues. Herbert Street and colleagues began a series of extensive studies on the nutrition of excised tomato root tips.

After 1950, rapid progress was made in plant tissue culture techniques. Also, many advancements were accomplished in the knowledge of plant development, especially in the area of the effects of PGRs. While intensive studies on the in vivo and in vitro effects of auxins continued, other classes of PGRs were now recognized. One such class, the cytokinins, emerged from the investigations of Skoog and associates on the nutritional requirements of tobacco callus, which extended to include study of the induction of bud formation on tobacco stem segments by adenine sulfate (Skoog and Tsui, 1951). These initial investigations led to the discovery of the cytokinin, kinetin, a cell division promoter, by Skoog and Carlos Miller. This research subsequently led to the discovery of other cytokinins during the latter half of the 1950s and into the 1960s. Intensive research then began with adenine, kinetin, and several other newly discovered cytokinins on their in vitro shoot-promoting effects for the rapid propagation of plants, especially the economically important horticultural and agronomic cultivars.

Meanwhile, according to Stowe and Yamaki (1957), the "western world" became familiar with a third class of PGR, the gibberellins, through the efforts of a group at Imperial Chemical Industries in Britain and a group at the USDA. These groups "discovered" research on gibberellins that had been conducted by many Japanese plant physiologists beginning with Kurosawa in what was then Formosa. After the "western world" became aware of the gibberellins in the early 1950s, research on the in vivo effects of gibberellins accelerated at a rapid pace worldwide. In vitro research with gibberellins was slow to begin in the late 1950s and increased substantially in the 1960s and 1970s.

A similar situation occurred with the two other classes of PGRs, each of which contains a single representative, ethylene and abscisic acid. Crocker et al. (1935) were the first to propose that ethylene is involved in fruit ripening. In vivo investigations in the 1960s confirmed the role of ethylene in fruit ripening, as first proposed in the 1930s, and also in early seedling growth, leaf abscission and epinasty, senescence, and other growth-promoting and inhibitory effects. In vitro studies with ethylene did not begin until the 1970s. The in vivo effects of abscisic acid on plant development were not recognized until the 1960s. Studies into the 1970s were directed toward leaf and fruit abscission and bud dormancy. Other plant growth-promoting and inhibiting studies soon followed. In vitro investigations with abscisic acid began in the 1970s with experiments involving zygotic and somatic embryogenesis.

By the mid 1950s, plant tissue culture methods had progressed to the point of making a major impact on research in plant developmental biology. Those investigators who had studied plant development over the previous 50 years using the conventional techniques of morphology, anatomy, physiology, biochemistry, cytology, and genetics progressively incorporated the "tool" of plant tissue culture into their research. Major breakthroughs of knowledge in plant development came sooner than would have been possible without the techniques of sterile culture. The progress also was hastened by innovations in laboratory equipment and supplies, and by improved methods of worldwide transportation and communication between developmental botanists around the world. In particular, the development of HEPA filters, which screen fungal spores and bacteria from air, made laminar flow transfer hoods possible. Laminar flow hoods became commonly available in the late 1960s to early 1970s and, finally, made sterile culture a routine task. Some of the European and American originators of the basic techniques of plant tissue culture from the 1930s and 1940s traveled around the world after the 1950s to many research laboratories as visiting botanists or to attend meetings, and became the "teachers" of the basic methods to numerous colleagues and students.

Concomitantly, another aspect of plant tissue culture greatly aided the increased research activity in plant development. The methods of obtaining plant callus tissue from several sources were well developed by the mid-1950s. The study of the causes of

mammalian cancer (especially human cancers) became very popular during this time period. It was logical, then, that plant callus development was equated to mammalian tumor development. Thus, research on plant callus and cell suspension cultures intensified in many research laboratories because several agencies that were interested in mammalian cancer research awarded generous grants to plant developmental botanists to study "plant cancers."Among these agencies were the American Cancer Society, the National Cancer Institute, the National Institutes of Health, and the U.S. Department of Health, Education and Welfare. A few notable pioneer investigators, among many, in the study of plant callus and cell suspensions in the early 1950s to the mid-1960s included Morel and Wetmore (1951), Henderson and Bonner (1952), Steward et al. (1952; 1954; 1958), Muir et al. (1954), Braun (1954), Nickell (1955), Partanen et al. (1955), Das et al. (1956), Nitsch and Nitsch (1956), Torrey (1957), Reinert (1958), Klein (1958), Bergmann (1960), and Murashige and Skoog (1962).

In the 1950s, the early prediction that somatic plant cells could undergo embryogenesis finally was validated by Steward et al. (1958) and Reinert (1958), who showed that somatic cells of carrot would differentiate into embryos when cultured within a proper nutrient — PGR regime. The ability to regenerate plants from single somatic cells through such a "normal" developmental process was envisioned to have great applications in propagation and genetic engineering (see below). Nonzygotic embryogenesis now has been demonstrated in most species of higher plants that have been tested (see Chapter nineteen).

In the early 1960s, Murashige completed a study while working in Skoog's laboratory leading to a commercial application for tissue culture. A culture medium developed by Murashige was originally devised for the rapid growth and bioassays with tobacco callus (Murashige and Skoog, 1962). However, research by several nurseries in California and elsewhere, through the advice of Murashige and knowledge of the work of Morel on orchid propagation, showed that practical shoot tip propagation of several ornamental plants could be accomplished on Murashige and Skoog medium (1962). Today, the ornamental plant industry depends heavily on tissue and organ culture micropropagation to supply high-quality, low-cost stocks of many species (see Chapters eight, ten, and forty-two).

Reports concerning the recovery of plants from haploid cells began to appear in the 1960s (see Chapter twenty-nine). The first successes were obtained with *Datura* (Guha and Maheshwari, 1966) and tobacco (Bourgin and Nitsch, 1967). This discovery received significant attention from plant breeders, since plants recovered from doubled haploid cells are homozygous and express all recessive genes, making them ideal pure breeding lines. Haploid-based breeding programs now are in place for several major agronomic crops.

Plant protoplasts were isolated and began to be cultured in the 1960s (Cocking, 1960). The removal of the plant cell wall allowed many novel experiments on membrane transport to be undertaken and, with totipotent protoplasts, allowed the somatic hybridization of sexually incompatible species to be accomplished. Somatic hybridization now has been used successfully in a number of cultivar development programs (Chapter twenty-six). In addition, the uptake of DNA into the plant cell was facilitated by removal of the wall, leading to the first reports of plant genetic transformation (Chapter thirty-one and below).

Perhaps the greatest stimulus and change in the direction of plant cell and tissue culture research occurred after the discovery of restriction endonuclease enzymes in the early 1970s. These enzymes cleave DNA molecules at predictable sites and allow specific genes to be removed, modified, and inserted into other DNA strands. The watershed of technological development that occurred after this breakthrough, and still is occurring today, has provided the basic tools needed for genetic engineering (Chapter thirty-one). The term "biotechnology" was coined to denote this new research field. The availability of totipotent plant cells that, conceivably, could be altered by insertion of specific genes caused a revolution in plant research, because the obvious implications to agriculture of

such genetically altered plants were so great. The potential commercial value of such genetically modified (or GM) plants attracted an unprecedented amount of industrial and investor interest such that, in the late 1970s and 1980s, a number of new plant biotechnology-based companies sprang up around the world, but primarily in the U.S. Some of these companies exist today, but many either failed or were absorbed by larger companies.

Since the publication of the first edition of *Plant Tissue Culture Concepts and Laboratory Exercises* in 1996, a renaissance in agriculture has occurred due to the commercialization of GM crops. Establishment of GM crops has been very rapid; to document growth of this new sector, the following statistics were gleaned from news reports from the past year. In 1995, there were no commercial plantings of GM crops. However, out of 80 million acres of corn planted yearly in the U.S., 400,000 acres of GM corn were planted in 1996, increasing to 3 million acres in 1997 and 17 million acres in 1998. Similarly, of 71 million acres of soybean planted in 1997, 20 million acres were genetically modified and some estimates are that nearly 100% of the soybean crop will be composed of GM varieties within the next few years. Approximately 45% of the U.S. 1998 cotton crop was genetically modifed. Corn and soybean account for about 75% of all GM crops planted, followed by cotton (12%), canola (11%), and potato (<1%). While almost 75% of all GM crops are grown in the U.S., production is increasing in Argentina, Canada, Mexico, Spain, France and South Africa. Although China grows GM crops, data as to the extent are not available.

In 1998, over 99% of the GM crop acreage was devoted to just two transgenic traits, herbicide and insect resistance (Bt). Less than 1% of GM acreage was devoted to "quality" traits, such as controlled ripening and enhanced oil profiles. Globally, herbicide-resistant soybean dominates GM crop acreage (52%), followed by Bt corn (24%). The success of these crops is evident in that it is estimated that $200 million worth of pesticide was eliminated by implementation of insect-resistant GM crops alone. Herbicide usage has dropped because applications can be delayed until weeds are commingled with the crop plant and are most susceptible.

When considering these GM crops, along with other additives in use today, such as enzymes from microorganisms in processed products like soft drinks, cakes, cheese, bread, fish, and meats, up to 90% of common foodstuffs already may contain GM components. This rapid integration of GM crops into the food supply has caused concern in certain parts of the world. The concern is based on the perception that there has been inadequate testing of GM crops and centers on several issues, including the following: (1) unwanted spread of transgenes into wild species by pollination, resulting in, for example, herbicide-resistant weeds; (2) development of resistant insects, for example, due to overuse of Bt crops; (3) potential for increased use of herbicides in herbicide-resistant GM crop production; (4) human health concerns, for example, transfer of antibiotic resistance genes from digested GM food to bacteria in the gut, and possible unrecognized toxicity of transgenic proteins. While controversial, most experts consider such concerns to be overreaction and often politically motivated, since GM crops already have been subjected to rigorous testing. Currently, several countries are considering multiyear moratoriums on the planting of GM crops. However, it is expected that the overall benign impact of GM crops on the environment, including reduction of chemical inputs, along with consumer recognition of resultant increased quality of foodstuffs will be combined with better education to solve these concerns.

The use of plant tissue culture technology to enable the development of GM crops illustrates its key, but often overlooked, importance in our lives. Plant tissue culture also is an increasingly important tool for advanced studies of plant development, especially with the integration of molecular biological techniques. However, as will become evident throughout this book, much remains to be learned in terms of the basic methods and procedures needed for efficient manipulation of plants in vitro.

Literature cited

Ball, E. 1946. Development in sterile culture of stem tips and subadjacent regions of *Tropaeolum majus* and of *Lupinus albus* L. *Amer. J. Bot.* 33:301–318.

Bergmann, L. 1960. Growth and division of single cells of higher plants in vitro. *J. Gen. Physiol.* 43:841–851.

Bhojwani, S. S., V. Dhawan, and E. C. Cocking. 1986. *Plant Tissue Culture: A Classified Bibliography.* p. 789. Elsevier Science Publishers, Inc., New York.

Bourgin, J. P. and J. P. Nitsch. 1967. Obtention de *Nicotiana* haploides a partir d'etamines cultivees in vitro. *Ann. Physiol. Veg.* 9:377–382.

Braun, A.C. 1954. Studies on the origin of the crown gall tumor cell. In: Abnormal and Pathological Plant Growth, Brookhaven National Lab. Symp. No. 6:115–127.

Camus, G. 1943. Sur le greffage des bourgeons d'endive sur des fragments de tissues cultives in vitro. *C. R. Acad. Sci.* 137:184–185.

Cocking, E. C. 1960. A method for the isolation of plant protoplasts and vacuoles. *Nature* 187:962–963.

Crocker, W., A. E. Hitchcock, and P. W. Zimmerman. 1935. Similarities in the effects of ethylene and the plant auxins. *Contributions Boyce Thompson Inst.* 7:231–248.

Das, N.K., K. Patau, and F. Skoog. 1956. Initiation of mitosis and cell division by kinetin and indoleacetic acid in excised tobacco pith tissue. *Physiol. Plant.* 9:640–651.

Dormer, K.J. and H.E. Street. 1949. The carbohydrate nutrition of tomato roots. *Ann. Bot. N.S.* 13:199–217.

Duhamet, L. 1939. Action de l'heteroauxine sur la croissance de racines isolees de *Luipinus albus*. *C. R. Acad. Sci.* 208:1838–1840.

Durosawa, E. 1926. Experimental studies on the secretions of *Fusarium heterosporum* on rice plants. *Trans. Nat. Hist. Soc. Formosa* 16:123-127.

Gautheret, R. J. 1934. Culture de tissu cambial. *C. R. Acad. Sci.* 198:2195–2196.

Gautheret, R. J. 1939. Sur la possibilite de realiser la culture indefinie des tissus de tubercules de carotte. *C. R. Acad. Sci.* 208:118–120.

Guha, S. and S. C. Maheshwari. 1966. Cell division and differentiation in the pollen grains of *Datura* in vitro. *Nature* 212:97–98.

Hannig, E. 1904. Zur physiologie pflanzlicher embryonen. I. Uber die kultur von cruciferen-embryonen ausserhalb des embryosacks. *Bot. Ztg.* 62:45–80.

Henderson, J.H.M. and J. Bonner. 1952. Auxin metabolism in normal and crown gall tissue of sunflower. *Amer. J. Bot.* 39:444–451.

Hildebrandt, A.C., A.J. Riker, and B.M. Duggar. 1946. The influence of the composition of the medium on growth in vitro of excised tobacco and sunflower tissue culture. *Amer. J. Bot.* 33:591–597.

Hogland, D.R. and D.I. Arnon. 1938. The Water Culture Method for Growing Plants Without Soil. Calif. Agric. Exp. Stat. Circ. 347, Berkeley, CA.

Klein, R.M. 1958. Activation of metabolic systems during tumor cell formation. *Proc. Natl. Acad. Sci. (U.S.A.)* 44:349–354.

Kotte, W. 1922. Kulturversuche mit isolierten wurzelspitzen. *Beitr. Allg. Bot.* 2:413–434.

Krikorian, A.D. and D.L. Berquam. 1969. Plant cell and tissue culture — the role of Haberlandt. *Bot. Rev.* 35:59-88.

La Rue, C. D. 1936. The growth of plant embryos in culture. *Bull. Torrey Bot. Club* 63:365- 382.

Maheshwari, P. 1950. *An Introduction to the Embryology of Angiosperms*. p. 453. McGraw-Hill, New York.

Morel, G. 1944. Le developpement du mildou sur des tissues de vigne cultives in vitro. *C. R. Acad. Sci.* 218:50-52.

Morel, G. 1948. Recherches sur la culture associee de parasites obligatoires dt de tissus vegetaux. *Ann. Epiphyt. N. S.* 14:123-234.

Morel, G. 1965. Clonal propagation of orchids by meristem culture. *Cymbidium Soc. News* 20:3-11.

Morel, G. and R.H. Wetmore. 1951. Fern Callus Tissue cultures. *Amer. J. Bot.* 39:141–143.

Muir, W.H., A.C. Hildebrandt, and A.J. Riker. 1954. Plant tissue cultures produced from single isolated cells. *Science* 119:877–878.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15:473–497.

Nickell, L.G. 1955. Nutrition of pathological tissues caused by plant viruses. *Ann. Biol.* 31:107–121.

Nitsch, J.P. and C. Nitsch. 1956. Auxin dependent growth of excised *Helianthus tuberosus* tissues. *Amer. J. Bot.* 43:839–851.

Nobecourt, P. 1937. Cultures en serie de tissus vegetaux sur milieu artificiel. *C. R. Seanc. Soc. Biol.* 205:521–523.

Partanen, C.R., I.M. Sussex, and T.A. Steeves. 1955. Nuclear behavior in relation to abnormal growth in fern prothalli. *Amer. J. Bot.* 42:245–256.

Reinert, J. 1958. Morphogenese und ihre kontrolle an gewebekulturen aus carotten. *Naturwiss.* 45:344–345.

Robbins, W. J. 1922. Cultivation of excised root tips and stem tips under sterile conditions. *Bot. Gaz.* 73:376–390.

Skoog, F. 1944. Growth and organ formation in tobacco tissue cultures. *Amer. J. Bot.* 31:19–24.

Skoog, F. and C.O. Miller. 1957. Chemical regulation of growth and organ formation in plant tissues cultured in vitro. *Symp. Soc. Exp. Biol.* 11:118–131.

Skoog, F. and C. Tsui. 1951. Growth substances and the formation of buds in plant tissues. In: *Plant Growth Substances.* Skoog, F., Ed. pp. 263–285. University of Wisconsin Press, Madison.

Steward, F.C. 1968. *Growth and Organization in Plants.* p. 564. Addison-Wesley Publishing, Reading, MA.

Steward, F.C. and S.M. Caplin. 1954. The growth of carrot tissue explants and its relation to the growth factors present in coconut milk. I(A). The development of the quantitative method and the factors affecting the growth of carrot tissue explants. *Ann. Biol.* 30:385–394.

Steward, F.C., S.M. Caplin, and F.K. Millar. 1952. Investigations on growth and metabolism of plant cells. I. New techniques for the investigation of metabolism, nutrition and growth of undifferentiated cells. *Ann. Bot. N. S.* 16:57–77.

Steward, F.C., M.O. Mapes, and M.J. Smith. 1958. Growth and organized development of cultured cells. I. Growth and division of freely suspended cells. *Amer. J. Bot.* 45:693–703.

Stowe, B. B. and T. Yamaki. 1957. The history and physiological action of the gibberellins. *Annu. Rev. Plant Physiol.* 8:181–216.

Street, H.E. and J.S. Lowe. 1950. The carbohydrate nutrition of tomato roots. II. The mechanism of sucrose absorption by excised roots. *Ann. Bot. N.S.* 14:307–329.

Torrey, J.G. 1957. Cell division in isolated single plant cells in vitro. *Proc. Natl. Acad. Sci.* (*U.S.A.*) 43:887–891.

Van Overbeek, J., M.E. Conklin, and A.F. Blakeslee. 1942. Cultivation in vitro of small *Datura* embryos. *Amer. J. Bot.* 29:472–477.

Went, F.W. and K.V. Thimann. 1937. *Phytohormones.* p. 294. Macmillan, New York.

Wetmore, R.H. 1954. The use of in vitro cultures in the investigation of growth and differentiation in vascular plants. In: *Abnormal and Pathological Plant Growth,* Brookhaven National Lab. Symp. No. 6:22–40.

White, P. R. 1934. Potentially unlimited growth or excised tomato root tips in liquid medium. *Plant Physiol.* 9:585–600.

White, P. R. 1937. Vitamin B_1 in the nutrition of excised tomato roots. *Plant Physiol.* 12:803–811.

White, P. R. 1939. Potentially unlimited growth of excised plant callus in an artificial medium. *Amer. J. Bot.* 26:59–64.

Part III

Supporting methodologies

chapter three

Getting started with tissue culture — media preparation, sterile technique, and laboratory equipment

Caula A. Beyl

A plant tissue culture laboratory has several functional areas, whether it is designed for teaching or research and no matter what its size or how elaborate it is. It has some elements similar to a well-run kitchen and other elements that more closely resemble an operating room. There are areas devoted to preliminary handling of plant tissue destined for culture, media preparation, sterilization of media and tools, a sterile transfer hood or 'clean room' for aseptic manipulations, a culture growth room, and an area devoted to washing and cleaning glassware and tools (see Chapter forty-two). The following chapter will serve as an introduction to what goes into setting up a tissue culture laboratory — what supplies and equipment are necessary and some basics concerning making stock solutions, calculating molar concentrations, making tissue culture media, preparing a transfer (sterile) hood, and culturing various cells, tissues, and organs.

Equipment and supplies for a tissue culture teaching laboratory

Ideally, there should be enough bench area to allow for both preparation of media and storage space for chemicals and glassware. In addition to the usual glassware and instrumentation found in laboratories, a tissue culture laboratory needs an assortment of glassware, which may include graduated measuring cylinders, wide-necked Erlenmeyer flasks, medium bottles, test tubes with caps, petri dishes, volumetric flasks, beakers, and a range of pipettes. In general, glassware should be able to withstand repeated autoclaving. Baby food jars are an inexpensive alternative tissue culture container well suited for teaching. Ample quantities can be obtained by preceding the recycling truck on its pickup day (provided you are not embarrassed by the practice). Some tissue culture laboratories find presterilized disposable culture containers and plastic petri dishes to be convenient, but the cost may be prohibitive for others on a tight budget. There are also reusable plastic containers available but their longevity and resistance to wear, heat, and chemicals varies considerably.

0-8493-2029-1/00/$0.00+$.50

It is also good to stock metal or wooden racks to support culture tubes both for cooling and later during their time in the culture room, metal trays (such as cafeteria trays) and carts for transport of cultures, stoppers and various closures, nonabsorbent cotton, cheese cloth, foam plugs, metal or plastic caps, aluminum foil, Parafilm™, and plastic wrap.

To teach tissue culture effectively, some equipment is necessary such as a pH meter, balances (one analytical to four decimal places and one analytical to two decimal places), bunsen burners, alcohol lamps or electric sterilizing devices, several hot plates with magnetic stirrers, a microwave oven for rapid melting of large volumes of agar medium, a compound microscope and hemocytometer for cell counting, a low-speed centrifuge, stereomicroscopes (ideally with fiber optic light sources), large (10- or 25-L) plastic carboys to store high-quality (purity) water, a fume hood, an autoclave (or, at the very least, a pressure cooker), and a refrigerator to store media, stock solutions, plant growth regulators (PGRs), etc. A dishwasher is useful, but a large sink with drying racks, pipette and acid baths, and a forced air oven for drying glassware will also work. Also, deionized distilled water for the final rinsing of glassware is needed. Aseptic manipulations and transfers are done in multistation laminar flow hoods (one for each pair of students).

Equipment used in the sterile transfer hood usually includes a spray bottle containing 70% ethanol, spatulas (useful for transferring callus clumps), forceps (short, long, and fine-tipped), scalpel handles (#3), disposable scalpel blades (#10 and #11), a rack for holding sterile tools, a pipette bulb or pump, bunsen burner, alcohol lamp or other sterilizing device, and a sterile surface for cutting explants (see below). If necessary for the experimental design, uniform-sized leaf explants can be obtained using a sterile cork borer (see Chapter four).

There are a number of options for providing a sterile surface for cutting explants. A previously autoclaved stack of paper towels wrapped in aluminum foil is effective and as each layer becomes messy, it can be peeled off and the next layer beneath it used (Figure 3.1). Others prefer reusable surfaces such as ceramic tiles (local tile retailers are quite generous and will donate samples), metal commercial ashtrays, or glass petri dishes (100 × 15 mm). Sterile plastic petri dishes also can be used but the cost may outweigh the advantages. A container is needed to hold the alcohol used for flaming instruments, if flame sterilization is used. An ideal container for this purpose is a slide staining Coplin jar with a small wad of cheesecloth at the bottom to prevent breakage of the glass when tools are dropped in. It has the advantage that it is heavier glass and since the base is flared, it is not prone to tipping over. Other containers can also serve the same purpose such as test tubes in a rack or placed in a flask or beaker to prevent them from spilling. Plastic containers, which can catch fire and melt, never should be used to hold alcohol.

Water

High-quality water is a required ingredient of plant tissue culture media. Ordinary tap water contains cation, anions, particulates of various kinds, microorganisms, and gases that make it unsuitable for use in tissue culture media. Various methods are used to treat water including filtration through activated carbon to remove organics and chlorine, deionization or demineralization by passing water through exchange resins to remove dissolved ionized impurities, and distillation that eliminates most ionic and particulate impurities, volatile and nonvolatile chemicals, organic matter, and microorganisms. The process of reverse osmosis, which removes 99% of the dissolved ionized impurities, uses a semipermeable membrane through which a portion of the water is forced under pressure and the remainder containing the concentrated impurities is rejected. The most universally reliable method of water purification for tissue culture use is a deionization treatment

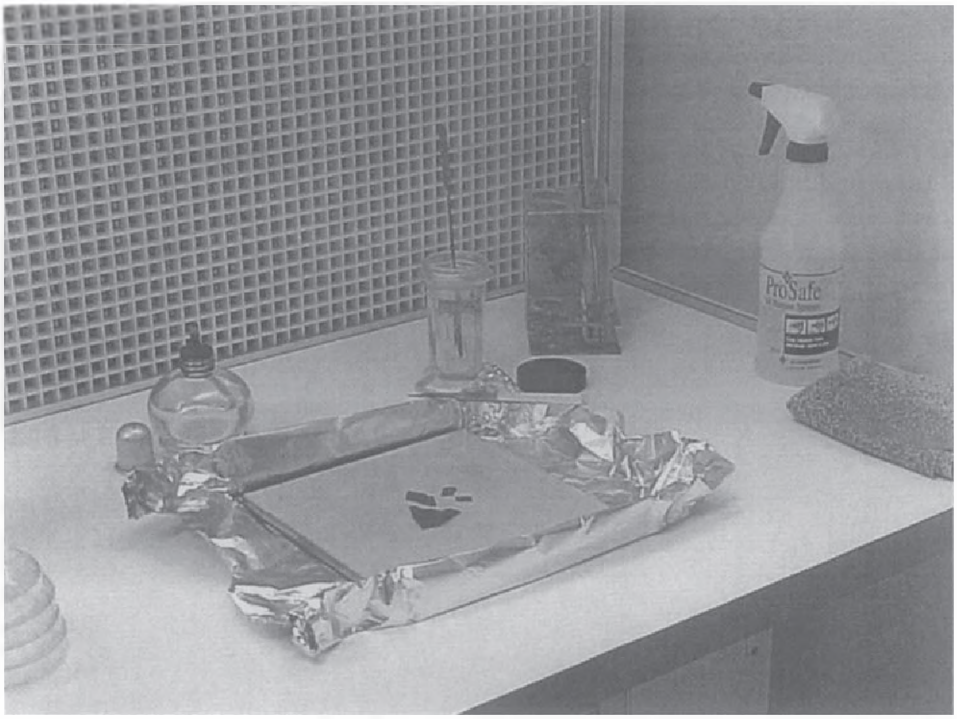

Figure 3.1 A typical layout of materials in the hood showing placement of the sterile tile work surface, an alcohol lamp, spray bottle containing 80% ethanol, a cloth for wiping down the hood, and two different kinds of tool holders — a glass staining (Coplin) jar and a metal rack for holding test tubes.

followed by one or two glass distillations, although simple deionization alone is sometimes successfully used. In some cases, newer reverse osmosis purifying equipment (Milli-RO™, Millipore™, RO pure™, Barnstead™, Bion™, Pierce™), combined with cartridge ion exchange, adsorption, and membrane filtering equipment, has replaced the traditional glass distillation of water.

The culture room

After the explants are plated on the tissue culture medium under the sterile transfer hood, they are moved to the culture room. It can be as simple as a room with shelves equipped with lights or as complex as a room with intricate climate control. Most culture rooms tend to be rather simple, consisting of cool white fluorescent lights mounted to shine on each shelf. Adjustable shelves are an asset and allow for differently sized tissue culture containers and for moving the light closer to the containers to achieve higher light intensities. Putting the lights on timers allows for photoperiod manipulation. Some cultures grow equally as well in dark or light. Temperatures of 26 to 28°C are usually optimum. Heat buildup can be a problem if the room is small, so adequate air conditioning is required. Good air flow also helps to reduce condensation occurring inside petri dishes or other vessels. Some laboratories purchase incubators designed for plant tissue culture. If a liquid medium is used, the culture room should be equipped with a rotary or reciprocal shaker to provide sufficient oxygenation. The optimal temperature, light, and shaker conditions vary depending on the plant species being cultured.

Characteristics of some of the more common tissue culture media

The type of tissue culture medium selected depends on the species to be cultured. Some species are sensitive to high salts or have different requirements for PGRs. The age of the plant also has an effect. For example, juvenile tissues generally regenerate roots more readily than adult tissues. The type of organ cultured is important; for example, roots require thiamine. Each desired cultural effect has its own unique requirements such as auxin (see below) for induction of adventitious roots and altering the cytokinin-to-auxin ratio for initiation and development of adventitious shoots.

Development of culture medium formulations was a result of systematic trial and experimentation. Table 3.1 gives a comparison of the composition of several of the most commonly used plant tissue culture media with respect to their components in milligrams/liter and molar units. Murashige and Skoog (1962) medium (MS) is the most suitable and most commonly used basic tissue culture medium for plant regeneration from tissues and callus. It was developed for tobacco based primarily on the mineral analysis of tobacco tissue. This is a "high salt" medium due to its content of K and N salts. Linsmaier and Skoog medium (1965) is basically Murashige and Skoog (1962) medium with respect to its inorganic portion, but only inositol and thiamine HCl are retained among the organic components. To counteract salt sensitivity of some woody species, Lloyd and McCown (1980) developed the woody plant medium (WPM).

Gamborg's B5 medium (Gamborg et al., 1968) was devised for soybean callus culture and has lesser amounts of nitrate and particularly ammonium salts than MS medium. Although B5 was originally developed for the purpose of obtaining callus or for use with suspension culture, it also works well as a basal medium for whole plant regeneration. Schenk and Hildebrandt (1972) developed SH medium for the callus culture of monocots and dicots. White's medium (1963), which was designed for the tissue culture of tomato roots, has a lower concentration of salts than MS medium. Nitsch's medium (Nitsch and Nitsch, 1969) was developed for anther culture and contains a salt concentration intermediate to that of MS and White's media.

Many companies (see Appendix I) sell packaged prepared mixtures of the better-known media recipes. These are easy to make because they merely involve dissolving the packaged mix in a specified volume of water. These can be purchased as the salts, the vitamins, or the entire mix with or without PGRs, agar, and sucrose. These are convenient, less prone to individual error, and make keeping stock solutions unnecessary. However, they are more expensive than making media from scratch.

Components of the tissue culture medium

Growth and development of explants in vitro are products of its genetics, surrounding environment, and components of the tissue culture medium, the last of which is easiest to manipulate to our own ends. Tissue culture medium consists of 95% water, macro- and micronutrients, PGRs, vitamins, sugars (because plants in vitro are often not photosynthetically competent), and sometimes various other simple-to-complex organic materials. All in all, about 20 different components are usually needed.

Inorganic mineral elements

Just as a plant growing in vivo requires many different elements from either soil or fertilizers, the plant tissue growing in vitro requires a combination of macro- and micronutrients. The choice of macro- and microsalts and their concentrations is species dependent. MS medium is very popular because most plants react to it favorably, however, it

Table 3.1 Composition of Five Commonly Used Tissue Culture Media in Milligrams per Liter and Molar Concentrations

Compounds	*Murashige* and Skoog	*Gamborg* B-5	WPM	*Nitsch and* Nitsch	*Schenk and* Hildebrandt	White
			Macronutrients in mg/L (mM)			
NH_4NO_3	1650 (20.6)	—	400 (5.0)	—	—	—
$NH_4H_2PO_4$	—	—	—	—	300 (2.6)	—
NH_4SO_4	—	134 (1.0)	—	—	—	—
$CaCl_2 \cdot 2H_2O$	332.2 (2.3)	150 (1.0)	96 (0.7)	166 (1.1)	151 (1.0)	—
$Ca(NO_3)_2 \cdot 4H_2O$	—	—	556 (2.4)	—	—	288 (1.2)
$MgSO_4 \cdot 7H_2O$	370 (1.5)	250 (1.0)	370 (1.5)	185 (0.75)	400 (1.6)	737 (3.0)
KCl	—	—	—	—	—	65 (0.9)
KNO_3	1900 (18.8)	2500 (24.8)	—	950 (9.4)	2500 (24.8)	80 (0.8)
K_2SO_4	—	—	990	—	—	—
KH_2PO_4	170 (1.3)	—	170 (1.3)	68 (0.5)	—	—
NaH_2PO_4	—	130.5 (0.9)	—	—	—	16.5 (0.12)
Na_2SO_4	—	—	—	—	—	200 (1.4)
			Micronutrients in mg/L (mM)			
H_3BO_3	6.2 (100)	3.0 (49)	6.2 (100)	10 (162)	5 (80)	1.5 (25)
$CoCl_2 \cdot 6H_2O$	0.025 (0.1)	0.025 (0.1)	—	—	0.1 (0.4)	—
$CuSO_4 \cdot 5H_2O$	0.025 (0.1)	0.025 (0.1)	0.25 (1)	0.025 (0.1)	0.2 (0.08)	0.01 (0.04)
Na_2EDTA	37.3 (100)	37.3 (100)	37.3 (100)	37.3 (100)	20.1 (54)	—
$Fe_2(SO_4)_3$	—	—	—	—	—	2.5 (6.2)
$FeSO_4 \cdot 7H_2O$	27.8 (100)	27.8 (100)	27.8 (100)	27.8 (100)	15 (54)	—
$MnSO_4 \cdot H_2O$	16.9 (100)	10.0 (59)	22.3 (132)	18.9 (112)	10.0 (59)	5.04 (30)
KI	0.83 (5)	0.75 (5)	—	—	0.1 (0.6)	0.75 (5)
$NaMoO_3$	—	—	—	—	—	0.001 (0.001)
$Na_2MoO_4 \cdot 2H_2O$	0.25 (1)	0.25 (1)	0.25 (1)	0.25 (1)	0.1 (0.4)	—
$ZnSO_4 \cdot 7H_2O$	8.6 (30)	2.0 (7.0)	8.6 (30)	10 (35)	1 (3)	2.67 (9)
			Organics in mg/L (mM)			
Myo-inositol	100 (550)	100 (550)	100 (550)	100 (550)	1000 (5500)	—
Glycine	2.0 (26.6)	—	2.0 (26.6)	2.0 (26.6)	—	3.0 (40)
Nicotinic acid	0.5 (4.1)	1.0 (8.2)	0.5 (4.1)	5 (40.6)	5.0 (41)	0.5 (4.1)
Pyridoxine HCl	0.5 (2.4)	0.1 (0.45)	0.5 (2.4)	0.5 (2.4)	0.5 (2.4)	0.1 (0.45)
Thiamin HCl	0.1 (0.3)	10.0 (30)	1.0 (3.0)	0.5 (1.5)	5.0 (14.8)	0.1 (0.3)
Biotin	—	—	—	0.2 (0.05)	—	—

may not necessarily result in the optimum growth and development for every species since the salt content is so high.

The macronutrients are required in millimolar (mM) quantities in most plant media. Nitrogen (N) is usually supplied in the form of ammonium (NH_4^+) and nitrate (NO_3^-) ions, although sometimes more complex organic sources, such as urea and amino acids like glutamine or casein hydrolysate, which is a complex mixture of amino acids and ammonium, are also used. Although most plants prefer NO_3^- to NH_4^+, the right balance of the two ions for optimum in vitro growth and development for the selected species may differ.

In addition to nitrogen, potassium, magnesium, calcium, phosphorus, and sulfur are provided in the medium as different components referred to as the macrosalts. $MgSO_4$

provides both magnesium and sulfur; $NH_4H_2PO_4$, KH_2PO_4, or NaH_2PO_4 provides phosphorus; $CaCl_2 \cdot 2H_2O$ or $Ca(NO_3)_2 \cdot 4H_2O$ provides calcium; and KCl, KNO_3, or KH_2PO_4 provides potassium. Chloride is provided by KCl and/or $CaCl_2 \cdot 2H_2O$.

Microsalts typically include boron (H_3BO_3), cobalt ($CoCl_2 \cdot 6H_2O$), iron (complex of $FeSO_4 \cdot 7H_2O$ and Na_2EDTA or rarely as $Fe_2(SO_4)_3$), manganese ($MnSO_4 \cdot H_2O$), molybdenum ($NaMoO_3$), copper ($CuSO_4 \cdot 5H_2O$), and zinc ($ZnSO_4 \cdot 7H_2O$). Microsalts are needed in much lower (micromolar, μM) concentrations than the macronutrients. Some media may contain very small amounts of iodide (KI), but sufficient quantities of many of the trace elements inadvertently may be provided because reagent-grade chemicals contain inorganic contaminants.

Organic compounds

Sugar is a very important part of any nutrient medium and its addition is essential for in vitro growth and development of the culture. Most plant cultures are unable to photosynthesize effectively for a variety of reasons including insufficiently organized cellular and tissue development, lack of chlorophyll, limited gas exchange and CO_2 in the tissue culture vessels, and less than optimum environmental conditions, such as low light. A concentration of 20 to 60 g/L sucrose (a disaccharide made up of glucose and fructose) is the most often used carbon or energy source, since this sugar is also synthesized and transported naturally by the plant. Other mono- or disaccharides and sugar alcohols such as glucose, fructose, sorbitol, and maltose may be used. The sugar concentration chosen is dependent on the type and age of the explant in culture. For example, very young embryos require a relatively high sugar concentration (>3%). For mulberry buds in vitro, fructose was found to be better than sucrose, glucose, maltose, raffinose, or lactose (Coffin et al., 1976). For apple, sorbitol and sucrose supported callus initiation and growth equally as well but sorbitol was better for peach after the fourth subculture (Oka and Ohyama, 1982).

Sugar (sucrose) that is bought from the supermarket is usually adequate, but be careful to get pure cane sugar as corn sugar is primarily fructose. Raw cane sugar is purified and according to the manufacturer's analysis consists of 99.94% sucrose, 0.02% water, and 0.04% other material (inorganic elements and also raffinose, fructose, and glucose). Nutrient salts contribute approximately 20 to 50% to the osmotic potential of the medium and sucrose is responsible for the remainder. The contribution of sucrose to the osmotic potential increases as it is hydrolyzed into glucose and fructose during autoclaving. This may be an important consideration when performing osmotic-sensitive procedures such as protoplast isolation and culture.

Vitamins are organic substances that are parts of enzymes or cofactors for essential metabolic functions. Of the vitamins, only thiamine (vitamin B_1 at 0.1 to 5.0 mg/L) is essential in culture as it is involved in carbohydrate metabolism and the biosynthesis of some amino acids. It is usually added to tissue culture media as thiamine hydrochloride. Nicotinic acid, also known as niacin, vitamin B_3, or vitamin PP, forms part of a respiratory coenzyme and is used at concentrations between 0.1 and 5 mg/L. MS medium contains thiamine HCl as well as two other vitamins, nicotinic acid and pyridoxine (vitamin B_6) in the HCl form. Pyridoxine is an important coenzyme in many metabolic reactions and is used in media at concentrations of 0.1 to 1.0 mg/L. Biotin (vitamin H) is commonly added to tissue culture media at 0.01 to 1.0 mg/L. Other vitamins that are sometimes used are folic acid (vitamin M, 0.1 to 0.5 mg/L), riboflavin (vitamin B_2, 0.1 to 10 mg/L), ascorbic acid (vitamin C, 1 to 100 mg/L), pantothenic acid (vitamin B_5, 0.5 to 2.5 mg/L), tocopherol (vitamin E, 1 to 50 mg/L), and para-aminobenzoic acid (0.5 to 1.0 mg/L).

Inositol is sometimes characterized as one of the B complex vitamin group but it is really a sugar alcohol involved in the synthesis of phospholipids, cell wall pectins, and

membrane systems in cell cytoplasm. It is added to tissue culture media at a concentration of about 0.1 to 1.0 g/L and has been demonstrated to be necessary for some monocots, dicots, and gymnosperms.

In addition, other amino acids are sometimes used in tissue culture media. These include L-glutamine, asparagine, serine, and proline, which are used as sources of reduced organic nitrogen, especially for inducing and maintaining somatic embryogenesis (see Chapter nineteen). Glycine, the simplest amino acid, is a common additive since it is essential in purine synthesis and is a part of the porphyrin ring structure of chlorophyll.

Complex organics are a group of undefined supplements such as casein hydrolysate, coconut milk (the liquid endosperm of the coconut), orange juice, tomato juice, grape juice, pineapple juice, sap from birch, banana puree, etc. These compounds are often used when no other combination of known defined components produces the desired growth or development. However, the composition of these supplements is basically unknown and may also vary from lot to lot causing variable responses. For example, the composition of coconut milk (used at a dilution of 50 to 150 ml/L), a natural source of the PGR, zeatin (see below), not only differs between young and old coconuts, but also between coconuts of the same age.

Some complex organic compounds are used as organic sources of nitrogen such as casein hydrolysate, a mixture of about 20 different amino acids and ammonium (0.1 to 1.0 g/L), peptone (0.25 to 3.0 g/L), tryptone (0.25 to 2.0 g/L), and malt extract (0.5 to 1.0 g/L). These mixtures are very complex and contain vitamins as well as amino acids. Yeast extract (0.25 to 2.0 g/L) is used because of the high concentration and quality of B vitamins.

Polyamines, particularly putrescine and spermidine, are sometimes beneficial for somatic embryogenesis. Polyamines are also cofactors for adventitious root formation. Putrescine is capable of synchronizing the embryogenic process of carrot.

Activated charcoal is useful for absorption of the brown or black pigments and oxidized phenolics. It is incorporated into the medium at concentrations of 0.2 to 3.0% (w/v). It is also useful for absorbing other organic compounds including PGRs such as auxins and cytokinins, vitamins, iron, and zinc chelates (Nissen and Sutter, 1990). Carry-over effects of plant growth regulators are minimized by adding activated charcoal when transferring explants to media without PGRs. Another feature of using activated charcoal is that it changes the light environment by darkening the medium so it can help with root formation and growth. It may also promote somatic embryogenesis and may enhance growth and organogenesis of woody species.

Leached pigments and oxidized polyphenolic compounds and tannins can greatly inhibit growth and development. These are formed by some explants as a result of wounding. If charcoal does not reduce the inhibitory effects of polyphenols, addition of polyvinylpyrrolidone (PVP, 250 to 1000 mg/L), or antioxidants such as citric acid, ascorbic acid, or thiourea can be tested.

Plant growth regulators

PGRs exert dramatic effects at low concentrations (0.001 to 10 μM). They regulate the initiation and development of shoots and roots on explants and embryos on semisolid or in liquid medium cultures. They also stimulate cell division and expansion. Sometimes a tissue or an explant is autotrophic and can produce its own supply of PGRs. Usually PGRs must be supplied in the medium for growth and development of the culture.

The most important classes of the PGRs used in tissue culture are the auxins and cytokinins. The relative effects of auxin and cytokinin ratio on morphogenesis of cultured tissues were demonstrated by Skoog and Miller (1957) and still serve as the basis for plant tissue culture manipulations today. Some of the PGRs used are hormones (naturally

synthesized by higher plants) and others are synthetic compounds. PGRs exert dramatic effects depending on the concentration used, the target tissue, and their inherent activity even though they are used in very low concentrations in the media (from 0.1 to 100 μM). The concentrations of PGRs are typically reported in milligrams/liter or in micromolar units of concentration. Comparisons of PGRs based on their molar concentrations are more useful because the molar concentration is a reflection of the actual number of molecules of the PGR per unit volume (see Table 3.2).

Auxins play a role in many developmental processes, including cell elongation and swelling of tissue, apical dominance, adventitious root formation, and somatic embryogenesis. Generally, when the concentration of auxin is low, root initiation is favored; and when the concentration is high, callus formation occurs. The most common synthetic auxins used are 1-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), and 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid (picloram). Naturally occurring indoleacetic acid (IAA) and indolebutyric acid (IBA) are also used. IBA was once considered synthetic, but has also been found to occur naturally in many plants including olive and tobacco (Epstein et al., 1989). Both IBA and IAA are photosensitive so that stock solutions must be stored in the dark. IAA is also easily broken down by enzymes (peroxidases and IAA oxidase). IAA is the weakest auxin and is typically used at concentrations between 0.01 and 10 mg/L. The relatively more active auxins such as IBA, NAA, 2,4-D, and picloram are used at concentrations ranging from 0.001 to 10 mg/L. 2,4-D and picloram are examples of auxins used primarily to induce and regulate somatic embryogenesis.

Cytokinins promote cell division and stimulate initiation and growth of shoots in vitro. The cytokinins most commonly used are zeatin, dihydrozeatin, kinetin, benzyladenine, thidiazuron, and 2-iP. In higher concentrations (1 to 10 mg/L) they induce adventitious shoot formation, but inhibit root formation. They promote axillary shoot formation by opposing apical dominance regulated by auxins. Benzyladenine (BA) has significantly stronger cytokinin activity than the naturally occurring zeatin. However, a concentration of 0.05 to 0.1 μM thidiazuron, a diphenyl-substituted urea, is more active than 4 to 10 μM BA, but thidiazuron may inhibit root formation, causing difficulties in plant regeneration. Adenine (used at concentrations of 2 to 120 mg/L) is occasionally added to tissue culture media and acts as a weak cytokinin by promoting shoot formation.

Gibberellins are less commonly used in plant tissue culture. Of the many gibberellins thus far described, GA_3 is the most often used, but it is very heat sensitive (after autoclaving 90% of the biological activity is lost). Typically it is filter sterilized and added to autoclaved medium after it has cooled. Gibberellins help to stimulate elongation of internodes and have proved to be necessary for meristem growth for some species.

Abscisic acid is not normally considered an important PGR for tissue culture except for somatic embryogenesis and in the culture of some woody plants. For example, it promotes maturation and germination of somatic embryos of caraway (Ammirato, 1974) and spruce (Roberts et al., 1990).

Organ and callus cultures are able to produce the gaseous PGR, ethylene. Since culture vessels are almost entirely closed, ethylene can sometimes accumulate. Many plastic containers also contribute to ethylene content in the vessel. There are contrasting reports in the literature concerning the role played by ethylene in vitro. It appears to influence embryogenesis and organ formation in some gymnosperms. Sometimes in vitro growth can be promoted by ethylene. At other times, addition of ethylene inhibitors results in better initiation or growth. For example, the ethylene inhibitors, particularly silver nitrate, are used to enhance embryogenic culture initiation in corn. High levels of 2,4-D can induce ethylene formation.

Table 3.2 Plant Growth Regulators, Their Molecular Weights, Conversions of mg/L Concentrations into µM Equivalents, and Conversion µM Concentrations into mg/L Equivalents

			mg/L equivalents for these µM concentrations				µM equivalents for these mg/L concentrations			
Plant growth regulator	Abbreviation	M.W.	0.1	1.0	10.0	100.0	0.1	0.5	1.0	10.0
Abscisic acid	ABA	264.3	0.0264	0.264	2.64	26.4	0.38	1.89	3.78	37.8
Benzyladenine	BA	225.2	0.0225	0.225	2.25	22.5	0.44	2.22	4.44	44.4
Dihydrozeatin	2hZ	220.3	0.0220	0.220	2.20	22.0	0.45	2.27	4.53	45.3
Gibberellic acid	GA3	346.4	0.0346	0.346	3.46	34.6	0.29	1.44	2.89	28.9
Indoleacetic acid	IAA	175.2	0.0175	0.175	1.75	17.5	0.57	2.85	5.71	57.1
Indolebutyric acid	IBA	203.2	0.0203	0.203	2.03	20.3	0.49	2.46	4.90	49.0
Potassium salt of IBA	K-IBA	241.3	0.0241	0.241	2.41	24.1	0.41	2.07	4.14	41.4
Kinetin	Kin	215.2	0.0215	0.215	2.15	21.5	0.46	2.32	4.65	46.7
Naphthaleneacetic acid	NAA	186.2	0.0186	0.186	1.86	18.6	0.54	2.69	5.37	53.7
Picloram	Pic	241.5	0.0242	0.242	2.42	24.2	0.41	2.07	4.14	41.4
Thidiazuron	TDZ	220.3	0.0220	0.220	2.20	22.0	0.45	2.27	4.54	45.4
Zeatin	Zea	219.2	0.0219	0.219	2.19	21.9	0.46	2.28	4.56	45.6
2-Isopentenyl adenine	2-ip	203.3	0.0203	0.203	2.03	20.3	0.49	2.46	4.92	49.2
2,4-Dichlorophenoxyacetic acid	2,4-D	221.04	0.0221	0.221	2.21	22.1	0.45	2.26	4.52	45.2