EXPERIMENTS IN
Plant Tissue Culture

Second Edition

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AND

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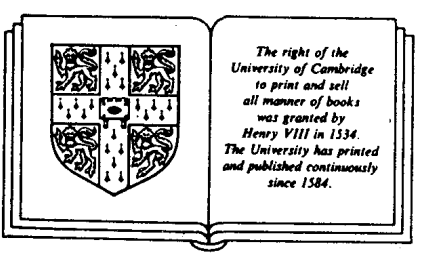

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FOREWORD

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 at Queen Elizabeth College, University of London. He gratefully acknowledges the cooperation of Professor P. B. Gahan, Head of the Biology Department.

We are grateful to all reviewers, colleagues, and students who have assisted us by their constructive criticisms 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.

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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.

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 physioiogical 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 hydrid
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-apex 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	aseptic 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

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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 "lepto-hormone," 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