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PLANT TISSUE AND CELL CULTURE

EDITED BY

H. E. STREET
D.Sc. (Lond.)
Botanical Laboratories,
University of Leicester,
England

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CONTRIBUTORS

P. A. AITCHISON *Department of Botany, University of Edinburgh, The King's Buildings, Mayfield Road, Edinburgh 9*

Y. P. S. BAJAJ *Institute of Plant Physiology and Cell Biology, The Free University of Berlin, Königin-Luise Str. 12–16a, 1 Berlin 33 (Dahlem), West Germany*

D. N. BUTCHER *Unit of Development Botany, Agricultural Research Council, University of Cambridge, 181A Huntingdon Road, Cambridge, CB3 0DY*

E. C. COCKING *Department of Botany, University of Nottingham, University Park, Nottingham, NG7 2RD*

J. M. DUNWELL *John Innes Institute, Colney Lane, Norwich, NOR 70F*

P. K. EVANS *Department of Biology, Medical and Biological Sciences Building, University of Southampton, Southampton, SO9 3TU*

D. S. INGRAM *Botany School, University of Cambridge, Downing Street, Cambridge, CB2 3EA*

P. J. KING *Friedrich Miescher Institut, Posfach 273, CH 4002, Basel, Switzerland*

A. J. MACLEOD *Department of Botany, University of Edinburgh, The King's Buildings, Mayfield Road, Edinburgh 9*

J. REINERT *Institute of Plant Physiology and Cell Biology, The Free University of Berlin, Königin-Luise Str. 12–16a, 1 Berlin 33 (Dahlem), West Germany*

H. E. STREET *Botanical Laboratories, Adrian Building, University of Leicester, Leicester, LE1 7RH*

N. SUNDERLAND *John Innes Institute, Colney Lane, Norwich, NOR 70F*

M. M. YEOMAN *Department of Botany, University of Edinburgh, The King's Buildings, Mayfield Road, Edinburgh 9*

B. ZBELL *Institute of Plant Physiology and Cell Biology, The Free University of Berlin, Königin-Luise Str. 12–16a, 1 Berlin 33 (Dahlem), West Germany*

PREFACE TO SECOND EDITION

The preparation of this Edition began 3 years after the 1st Edition went to press. Even in this relatively short period progress in some research fields covered was such that some Chapters (5, 9, 13, 14) had to be completely rewritten and others (Chapters 8, 11, 12) very extensively revised. Less extensive but nevertheless important revision of the other Chapters was called for. The extent of this revision is reflected in the List of References; this now cites 1530 publications (compared with 1103 in the 1st Edition) and of these 460 are dated 1973 or more recently.

In preparing this Edition I have not only been able to call again upon all my principle contributors but they have as before delivered their manuscripts promptly. Their co-operation and that of Robert Campbell (Blackwell Scientific Publications Ltd.) have made easy what seemed at first a very formidable task.

January 1977 H.E.Street

PREFACE TO FIRST EDITION

This book is an attempt to survey the rapid developments in plant tissue and cell culture techniques which have occurred in the last decade and to assess critically the contributions these techniques are now making to our knowledge of the growth, metabolism and differentiation of plant cells and of the factors controlling morphogenesis in vascular plants. Such a task has demanded the participation of a number of specialists and I feel most fortunate in the enthusiastic collaboration I have received from a group of authors each distinguished in the field of study he has covered. No attempt has been made to achieve any uniformity of approach or style in the separate chapters nor to completely eliminate overlap between them (this aspect is covered by numerous cross-references between chapters). Nevertheless, all the chapters seem to me to cover basic techniques and current researches in a way which not only will introduce established scientists, post-graduates and undergraduates to new fields of enquiry but will also provide details of current work (quite often previously unpublished work) of interest to those already using plant tissue and cell culture techniques in their work.

As Editor I should like to acknowledge my indebtedness not only to my co-authors but to Professor J.H.Burnett (General Editor of the Botanical Monographs Series) and to Robert Campbell (Blackwell Scientific Publications Ltd.). As will be seen in the legends of many text-figures and tables, we have also to acknowledge permission to publish new data and previously published data from many authors and journals. In the general preparation of the manuscript and particularly of the Literature Cited and Index I have been greatly helped by my secretary, Miss Daphne Roberts. Although many people have been involved in preparing the text-figures and plates, particular acknowledgement must be made for photography to Mr. G.G.Asquith (Illustration Service) and for line drawings to Mr. E.M.Singer, Miss S.Pearcey and Miss S.Duffey (Botanical Laboratories), all of the School of Biological Sciences, University of Leicester.

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INTRODUCTION

H. E. STREET

'The value of continually advancing technique is inestimable so long as it is not allowed to become an end in itself, and thus foster delusive industry of a pointless kind'
Agnes Arber—*The Mind and the Eye*, p. 13. University Press, Cambridge, 1954

The last decade has seen a very rapid rise in the number of plant scientists using the techniques of organ, tissue and cell culture in plant physiological researches. This is in part due to important developments and refinements of these techniques which now make possible an increasing range of reproducible and quantitative experiments involving plant cultures. It is also in part due to the demonstration, by a long sequence of pioneer investigations, that many problems in plant physiology otherwise inaccessible to study can be tackled by the imaginative use of plant culture techniques. Even more widespread use of the techniques is now probably inhibited primarily by a feeling that they are intrinsically difficult to carry out successfully and also that they are demanding of very special facilities and an exceptionally high level of skilled technical assistance. The main purpose of the present volume is therefore to describe clearly current techniques and to assess their potential value and current limitations. This should enable research workers to assess how far they could be of value in their own field of study and to make an informed judgement of the technical problems they are likely to encounter in their use.

There is, at present, in all plant culture work still a strong empirical element; a necessity to arrive at technical solutions by trial and error. In consequence the various protocols, presented in the chapters which follow, can only form a sound starting-point. For successful work with a particular plant material it may be necessary to vary manipulative procedures, methods of sterilization, composition of culture medium, conditions of incubation and so on. Therefore, following upon the various basic protocols are summaries of some of the variations in the basic procedure which have proved valuable with particular plants, and the references from which details of the variations can be obtained. It should therefore be possible for an investigator new to the

field to plan a systematic approach to the problem of establishing cultures from his chosen material. Nevertheless, even the best planned approach may fail; there are problems, seemingly formidable problems, still to be solved before it can be claimed that cultures can be successfully established from just any higher plant.

Each aspect of plant culture technique has evolved in an attempt to solve not only a technical problem but to open up or extend a line of biological investigation. This volume is therefore not simply a technical manual but an account of the present state of knowledge regarding a number of aspects of plant physiology to which the culture techniques have made a substantial and often a unique contribution. Here inevitably it has been necessary to select from a now very extensive literature, some of which could not be consulted in the original by the authors. The literature citations are therefore incomplete but nevertheless, it is hoped, properly representative of the main lines of enquiry.

SOME TERMS DEFINED

There is at present considerable confusion and lack of uniformity in the terminology of aseptic plant culture. The term 'plant tissue culture' although commonly used as a blanket phrase to cover all types of aseptic plant culture should now be used in a more restricted sense. It is possible to distinguish the following types of aseptic cultures of plant origin: The culture of seedlings or larger plants (*plant cultures*), of isolated mature or immature embryos (*embryo cultures*), of isolated plant organs (*organ cultures* including cultures derived from root tips, stem tips, leaf primordia, primordia or immature parts of flowers, immature fruits), of the tissues arising by proliferation from segments (explants) of plant organs (*tissue* or *callus cultures*), of isolated cells or very small cell aggregates remaining dispersed as they grow in liquid media (*suspension cultures*).

Tissue or callus cultures do not correspond with any normal tissue of the whole plant. The use of the term *callus cultures* derives from the fact that involved in their origin is proliferation induced in the explant by injury of cells caused by excision, suggesting that the tissue formed can be equated with wound callus. Such injury is, however, only one and in some cases probably not the principal cause of the proliferation; the removal of the explant from controls imposed upon its tissues by the whole plant and the provision to the explant of appropriate nutrients and growth-regulating substances may be among the determinative factors leading to proliferation (see Chapter 3). The established culture may differ very much from young wound callus. The alternative and possibly to be preferred description of such cultures as

tissue cultures arises from the fact that they can be derived from different tissues in the parent explant and can differ very markedly from one another (either as a consequence of their different specific or varietal origins or the conditions under which they are cultured) and from the primary wound callus in morphology, cellular structure, growth and metabolism. Such cultures always contain both dividing and non-dividing cells, and the non-dividing cells may be of one or several distinct cell types within the tissue mass. The term tissue culture can, therefore, be appropriately applied to any culture growing on solid medium (or attached to a substratum and fed with liquid medium) and which consists of many cells in protoplasmic continuity. It does not imply any structural or functional homogeneity of the constituent cells or equivalence with any normal plant tissue.

The term *suspension culture* is self-explanatory in so far as it implies cells and cell aggregates growing dispersed in a moving liquid medium. At present no such cultures consist entirely of separate cells (such a culture would be described as a free-cell suspension culture). Suspension cultures have also been termed *cell cultures* on the grounds that they represent a distinctly lower level of organization than tissue cultures; at least their free cells can properly be described as 'cells in culture'. The term cell culture has descriptive value even if it can be objected to on the grounds that cell aggregates are never absent and may transiently be very prominent even in the most highly dispersed suspensions currently in culture. The term cell culture has also been applied to work on the growth of single-cultured cells at least initially out of physical contact with other cells. Such work is preferably described as *single-cell cloning* (see Chapter 8).

Where root cultures are all derived from a single initial root tip or where a tissue culture (or suspension culture) derived from a single explant is maintained in culture by repeated subculture of a many-celled piece of the parent culture the term *clone* (root clone, tissue clone, suspension clone) seems appropriate (although the term *strain* is often used in this sense). Occasionally the tissue cultures of such a clone may not be of uniform morphology; they may show limited regions (sectors) differing in texture, colour and/or growth rate. By selective subculture of such sectors, the clone may be split to give two or more lines which appear to remain permanently distinct by propagation of the sector characters. These lines may then be termed *clonal variants*. The fact that such variants differ in recognizable characters does not necessarily imply that the variants are genetically distinct (though it may in fact be so). There is some evidence (almost entirely unpublished) that some such variants represent rather stable 'states of differentiation' which are potentially reversible by appropriate cultural treatments. It should also be borne in mind that the initial proliferation does not occur from a single cell of the primary explant nor even necessarily from a single tissue

of the explant. The primary tissue culture will, therefore, frequently contain cells of different previous histories or even of different levels of ploidy. The variant may therefore contain a different balance between the cell types present in the initial clone. Even when variants are not separated the cellular composition of the clone may alter as subculture proceeds by selection of cells at a growth advantage under the chosen cultural conditions.

To distinguish tissue clones as described above from clones derived from single cells the latter are described as *single-cell clones* and, where such clones (derived from a tissue clone) differ from one another, they can be described as *single-cell variants*. The term *mutant* rather than variant should be reserved for cases where there is an established genetic difference or where at least the new culture has arisen as a consequence of the application of an effective mutagenic treatment (preferably to single cells from which the mutant tissue culture has been developed).

It is important to distinguish between (1) culture of primary explants derived from plant organs (*cultured explants*), (2) culture for a single incubation period (passage) following excision of the newly proliferated tissue from the original organ explant (*1st passage cultures*), (3) culture through many successive passages (usually indicative of a potential for indefinite growth in culture provided an appropriate subculture procedure is adopted). Cultures of this last type have been described as continuous cultures. Since, however, they are propagated by successive batch cultures it is preferable to refer to them as *established or permanent cultures* and reserve the term *continuous cultures* for suspension cultures propagated over long periods of time without subculture by constant addition of new medium and a balancing harvesting of the suspension (see Chapters 4 and 11).

Tissue cultures are subcultured by transferring to new medium a fragment of the parent culture. Such a fragment has been termed an explant but it is preferable to term it an *inoculum* (the same term can then be applied to the aliquot of a suspension culture transferred to effect its subculture). The term *explant* should be reserved for the fragment of a plant or tissue (e.g. endosperm) used to initiate a culture clone.

The use of the term *aseptic* is preferable to sterile as a prefix—aseptic tissue culture—since such cultures are grown from surface-sterilized explants under aseptic conditions (conditions designed to exclude contaminants) and should be periodically tested for sterility (by microscopic examination and transfer to appropriately enriched media) but cannot be claimed at all times to be absolutely free from any contaminating organisms (see Chapter 2).

Organogenesis is used to describe the origin of shoot buds or roots from tissue cultures or suspension cultures. Plantlets can arise from these by the formation of adventitious roots from the shoot bud or of a shoot bud from tissue formed by proliferation at the base of the rootlet. *Embryogenesis* is used

to describe the origin of plantlets by a developmental pathway closely resembling the normal embryology from the fertilized ovum. That this development in culture involves somatic (body) cells and not the zygote can be indicated by using the term *embryoid* or *adventive embryo* rather than embryo. This does not preclude employing the recognized description of stages in embryology, e.g. proembryoid, globular embryoid, torpedo-shaped embryoid (see Chapter 12).

SCOPE OF THE PRESENT WORK

The present work is confined to a consideration of tissue (callus) and suspension (cell) cultures as defined above, and only those of either flowering plant or gymnosperm origin. The omission of organ cultures and embryo cultures does not imply that their potential has been exhausted, or that they are not being used in interesting current researches. Simply some restrictions of the field covered has been essential lest this book be too long. It is, however, pertinent to the omission both that the techniques of organ culture are not in a period of rapid change and development, and that those techniques and their more important contributions to botanical research have been covered in a number of recent review chapters (Butcher & Street 1964, Street & Henshaw 1966, Street 1969). The anther cultures discussed in Chapter 9 are not organ cultures; the immature anther is being used as an explant which, under appropriate conditions, can yield from its microspores haploid embryoids or a haploid tissue culture.

SOME LANDMARKS ON THE WAY

Haberlandt, in a frequently quoted paper published in 1902 (and now available in translation with an interesting commentary—Krikorian & Berquam 1969) stated clearly the desirability of culturing the isolated vegetative cells of higher plants: 'To my knowledge, no systematically organized attempts to culture isolated vegetative cells from higher plants in simple nutrient solutions have been made. Yet the results of such culture experiments should give some interesting insight into the properties and potentialities which the cell as an elementary organism possesses. Moreover, it would provide information about the inter-relationships and complementary influences to which cells within the multicellular whole organism are exposed.' Experiments along these lines had been started by Haberlandt in 1898, using single cells isolated from the palisade tissue of leaves, pith parenchyma, the epidermis and epidermal hairs of various plants. 'In my cultures . . . cell division was never observed. It will be the problem of future culture experiments to discover the conditions

under which isolated cells undergo division.' Haberlandt's failure did not deter others in his laboratory from attempts, over a number of years, at similar experiments but, although the cells in some cases remained alive for prolonged periods, and even expanded in the culture media, they did not divide to give rise to cell aggregates (Winkler 1902, Thielmann 1924, 1925, Küster 1928).

The great interest of Haberlandt's paper rests on its clear concept of the problem and its foresight as to what would be achieved. Although Haberlandt was working 30 years before the discovery of auxins, he was struck by Winkler's observations on the stimulation of ovule development and the swelling of ovaries which occurred when the pollen grains germinated and he suggested 'it would be worth while to culture together in hanging drops vegetative cells and pollen tubes, perhaps the latter would induce the former to divide'. He also proceeded as follows: 'One could also add to the nutrient solutions used an extract from vegetative apices or else culture the cells from such apices. One might also consider utilization of embryo sac fluids.' 'Without permitting myself to pose further questions, I believe in conclusion, that I am not making too bold a prediction if I point to the possibility that in this way, one could successfully cultivate artificial embryos from vegetative cells.'

Very little progress was made during the 30 years following Haberlandt's paper. Various workers in other laboratories reported further unsuccessful attempts to culture cells (Schmucker 1929, Scheitterer 1931, Pfeiffer 1931, 1933, La Rue 1933), and Kotté (1922a, b) and Robbins (1922a, b) reported some progress towards the culture of excised root tips. However, in 1934 the pioneer work on root culture reached fruition when White (1934) was able to report the establishment of an actively growing clone of tomato roots. In the same year Gautheret reported that pieces of cambium removed under aseptic conditions from *Salix capraea*, *Populus nigra* and other trees continued to proliferate for some months, giving rise to alga-like outgrowths if placed on the surface of a solidified medium containing Knop's solution, glucose and cysteine hydrochloride. White's (1937) discovery of the importance of the B vitamins for the growth of cultured roots and the increasing recognition of the importance of auxin (indol-3yl-acetic acid, IAA) in the control of plant growth (Went & Thimann 1937) led Gautheret (1937, 1938) to include these growth factors in his culture medium with the result that he obtained greatly enhanced but still limited growth of the *Salix* cambium. At this time Nobécourt (1937, 1938a, b) was obtaining some cell proliferation in culture using explants from carrot roots. Then, in 1939, Gautheret also reported studies on small explants (which included cambium and functional phloem) from carrot roots using a culture medium containing the modified inorganic salt mixture employed by Nobécourt: glucose, vitamin B_1 (thiamin),

cysteine hydrochloride and IAA. White, reviewing the field in 1941 wrote of Gautheret's work as follows: 'On 9 January 1939, Gautheret presented before the French Academy of Sciences the results of studies constituting a combination of his own previous work with that of Nobécourt. Using a Knop solution supplemented with Bertholot's mixture of accessory salts, glucose, gelatine, thiamin, cysteine hydrochloride and indole acetic acid . . . he had cultivated fragments of carrot . . . they showed little or no differentiation beyond the formation of occasional lignified cells . . . they grew slowly and without any indication of diminution of growth rate. The record, though brief, if taken with Gautheret's earlier work, is sufficient to justify the conclusion that he has obtained cultures satisfying both major criteria of a plant tissue culture—potentially unlimited growth and undifferentiated growth—so that there need be no further doubt as to the real success of his efforts.' The emphasis by White on the significance and success of Gautheret's work is particularly commendable for within months of Gautheret's paper, White (1939a) himself reported the formation of a similar tissue culture from the procambial tissue of segments of young stems of the hybrid *Nicotiana glauca* × *N. langsdorffi* and showed that it also could be repeatedly subcultured using a medium containing 0·5% agar but otherwise identical with that he had developed for the growth of his root clone of tomato. The basic technique of tissue culture described in these pioneer papers by Gautheret and White has subsequently resulted in the establishment of such cultures from many species.

Almost immediately, tissue cultures from more species were described (including some from bacteria-free crown-gall tissues—Braun & White 1943) and the cultures were submitted to anatomical study. Although young, actively growing cultures were found to consist of dividing cells (often localized in layers or nodules) and of parenchymatous non-dividing cells, the cultures as they aged showed an increasing degree of organization as exemplified by the development of primitive vascular tissue often composed entirely of tracheidal cells. Many studies on this histogenesis were undertaken but progress towards identification of the factors controlling such cellular differentiation had to wait till much later (Gautheret 1957, 1966). Nobécourt's pioneer studies on carrot had shown that tissue cultures could differentiate roots, and White (1939b) had described the development of leafy buds when his tissue culture of *N. glauca* × *N. langsdorffi* was transferred to liquid medium. Here again, however, no immediate progress was made towards identifying the factors controlling such organogenesis.

During the period from 1939–50, experimental work with root cultures drew attention to the role of vitamins in plant growth and advanced our knowledge of the shoot–root relationship (Street 1957, 1959, 1966a). In the history of plant tissue culture, however, it is a period not inaptly described by Honor Fell (1959) commenting on the first phase in the development of

animal tissue culture: 'When tissue culture first appeared on the biological horizon, great things were expected of it. The technique was so elegant, the growing cells were so aesthetically pleasing, that most people felt that tissue culture would immediately lead to major discoveries. Unfortunately, however, too many workers were attracted to the fashionable new field, who were not equipped with the necessary basic knowledge of cytology, physiology and biochemistry that would enable them to make practical use of the method once they had learned it, with the result that tissue culture became a closed world of its own and the mass of descriptive paper that appeared had little relation to current scientific issues.'

However, towards the end of this period and during the early 1950s a number of lines of enquiry were initiated which were to lead to a period of new interest and activity. The studies by Camus (1949) on the induction of vascular differentiation resulting from grafting buds into tissue culture masses led on to important studies on factors controlling vascular tissue differentiation by Wetmore & Sorokin (1955), Wetmore & Rier (1963) and Jeffs & Northcote (1967) (this is further discussed in Chapter 10). The work of Miller & Skoog (1953) on bud formation from cultured pith explants of tobacco led on to the discovery of kinetin (Miller, Skoog, Okumura, van Saltza & Strong 1956). In 1952, Steward initiated work on cultured carrot explants (Steward, Caplin & Millar 1952) which for the first time involved analysis of culture growth in quantitative terms and was to lead to the wide use of coconut milk as a nutrient, and to the discovery of embryogenesis (Steward 1958, Steward, Mapes & Mears 1958, Reinert 1958, 1959, Pilet 1961) (this is further discussed in Chapter 12). In 1953, Muir reported that if fragments of callus of *Tagetes erecta* and *Nicotiana tabacum* are transferred to liquid culture medium and the medium is agitated on a reciprocal shaker, then the callus fragments break up to give a suspension of single cells and cell aggregates and that this suspension can be propagated by subculture (Muir, Hildebrandt & Riker 1954). Similarly, in 1956, Steward and Shantz reported that the supernatant medium bathing their carrot root explants became turbid, due to the presence in it of free-floating cells and small groups of cells. Here again the suspension grew and could be serially subcultured. In 1956 Nickell reported that he had maintained for 4 years by serial subculture a suspension rich in free cells and derived from the hypocotyl of *Phaseolus vulgaris* (these techniques are further discussed in Chapter 4). It is from work with such cell suspensions, particularly involving their growth in continuous culture systems, that important advances have been made in our knowledge of the regulation of cell division, expansion and differentiation in plant cells (see Chapter 11).

Muir, in his Ph.D. thesis of 1953, reported a further important observation which was to open up the possibility of realizing Haberlandt's objective of culturing single vegetative cells. He carefully isolated, from his suspension

cultures and from friable calluses, both of transformed bacteria-free crown-gall tumour cells, uninjured single cells, and succeeded in obtaining from a small proportion of these cells growing tissue cultures (single-cell clones). This he did by placing the single cells on the upper surface of squares of filter paper whose lower surface made intimate contact with an actively growing 'nurse' crown-gall tissue culture (paper raft nurse technique) (see Fig. 8.1A, p. 208). This arrangement provided the single cells not only with the known nutrients of the culture medium (transmitted via the tissue culture) but also with growth factors synthesized in the massive tissue culture and essential for the induction of division in the isolated cell. Although Muir's success here was due to his choice of the right species and the use of crown-gall tumour cells (see Chapter 13) instead of normal cells, it is from these experiments that later techniques of obtaining single cell clones have developed. One of these techniques is that of agar plating first tested with cells from suspension cultures by Bergmann (1960). By filtering suspension cultures of *Nicotiana tabacum* var. 'Samsum' and *Phaseolus vulgaris* var. 'Early Golden Cluster' he obtained suspensions, 90% of the cells of which were free cells. He then incorporated such suspensions into 1 mm layers of solidified medium (0·6% agar) in petri dishes. The dishes were then sealed and incubated in diffused light. A proportion of the free cells divided and gave rise to visible colonies which could be built up into tissue cultures. This Bergmann technique, modified in a number of small but together important ways, is currently being used in a number of laboratories concerned with single-cell cloning (as discussed in Chapter 8).

In 1959 Melchers and Bergmann cultivated tissue derived from a haploid shoot of *Antirrhinum majus*. The tissue retained its haploid state during several subcultures but then increased in ploidy. Haploid tissue and suspension cultures are clearly of particular interest for those interested in studying mutations, and this subject has been activated again by the demonstration that haploid embryoids and haploid tissue can be obtained by using as explants tobacco anthers excised at the right stage of flower development (Bourgin & Nitsch 1967, Nakata & Tanaka 1968). The species whose anthers are known to behave in this way are very limited at present but if their number can be increased a wide range of haploid tissue cultures will become available. One possible way in which this range could be expanded is by the culture of isolated microspores (pollen as opposed to anther culture) since some success in embryoid development from cultured microspore suspensions of *Nicotiana tabacum* has already been reported (Nitsch 1974a). (This subject is further discussed in Chapter 9.)

The release of protoplasts from root tip cells using a fungal cellulase in 0·6 M sucrose was reported by Cocking in 1960. Protoplasts released by cell-wall degrading enzymes have now been prepared from many plant tissues,