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# Review of Cytology

EDITED BY

G. H. BOURNE
*St. George's University School of Medicine*
*St. George's, Grenada*
*West Indies*

J. F. DANIELLI
*Worcester Polytechnic Institute*
*Worcester, Massachusetts*

ASSISTANT EDITOR

K. W. JEON
*Department of Zoology*
*University of Tennessee*
*Knoxville, Tennessee*

SUPPLEMENT 11A

# Perspectives in Plant Cell and Tissue Culture

EDITED BY

INDRA K. VASIL
*Department of Botany*
*University of Florida*
*Gainesville, Florida*

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# List of Contributors

Numbers in parentheses indicate the pages on which the authors' contributions begin.

M. W. BAYLISS (113), *Imperial Chemical Industries Ltd., Corporate Laboratory, The Heath, Runcorn, Cheshire, England*

E. FORCHE* (1), *Department of Botany, University of Edinburgh, Edinburgh EH9 3JH, Scotland*

P. J. KING (25), *Friedrich Miescher-Institut, Postfach 273, 4002 Basel, Switzerland*

PÁL MALIGA (225), *Institute of Plant Physiology, Biological Research Center, H-6701 Szeged, Hungary*

RICHARD PHILLIPS (55), *Department of Botany and Microbiology, University College London, London WC1E 6BT, England*

TREVOR A. THORPE (71), *Department of Biology, University of Calgary, Calgary, Alberta T2N 1N4, Canada*

K. TRAN THANH VAN (175), *Laboratoire Phytotron, C.N.R.S., Gif-sur-Yvette 91190, France*

INDRA K. VASIL (145, 195), *Department of Botany, University of Florida, Gainesville, Florida 32611*

VIMLA VASIL (145), *Department of Botany, University of Florida, Gainesville, Florida 32611*

M. M. YEOMAN (1), *Department of Botany, University of Edinburgh, Edinburgh EH9 3JH, Scotland*

* Present address: GBF D-3300 Braunschweig, West Germany.

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# Preface

The understanding of plant growth, development, and differentiation remains one of the most basic and challenging problems in plant biology. Plant tissue culture techniques, among many other methodologies, provide a powerful and unique tool for the study of these problems. Serious efforts to grow plant tissues in culture began with the pioneering efforts of Gautheret, Nobecourt, and White in the late 1930s. Discovery of the cytokinins, itself a direct result of plant tissue culture studies, greatly stimulated research on the role of plant growth substances in cell division, differentiation, and morphogenesis, and led to the successful regeneration of plants from isolated tissues in many species. The regeneration of entire plants from isolated cells, in 1965, affirmed the totipotency of plant cells. Then, during the short span of 7 years, 1966–1972, three major developments took place in quick succession: regeneration of haploid plants from highly specialized gametophytic cells (microspores), regeneration of plants from isolated protoplasts, and finally, regeneration of somatic hybrid plants following protoplast fusion. It is these discoveries and developments, which together, form the basis of the current widespread interest in the utilization of plant tissue culture techniques for the study, resolution, and attainment of a wide variety of research problems and objectives, including large scale clonal propagation of desirable genotypes, elimination of viruses from plants or the selection of disease-resistant varieties, biosynthesis of pharmaceutically useful metabolites from plant cells in culture, preservation of germ plasm, isolation and characterization of mutant cell lines and their use in plant improvement, overcoming problems of sexual incompatibility in the production of desirable hybrids, and most importantly, the modification of the genetic content of plant cells by parasexual methods resulting in the production of improved and/or novel genotypes.

Some of these objectives have already been achieved during the last decade, and are being successfully exploited commercially, as the elimination of viruses and the large-scale clonal propagation of selected horticultural plants. Progress made in the pursuit of the remaining objectives is encouraging. I believe that a majority of these too can be achieved by the end of this century, provided the current level of interest is not only sustained but augmented, there is a substantial increase in the level of support for such research, and a greater degree of emphasis is placed on the study and the resolution of the basic problems currently hindering

full development and exploitation of these techniques. The present shortage of trained personnel will be remedied in the near future as a result of excellent courses and training workshops now being organized in many universities, and by many national and international organizations.

The current two companion volumes on *Perspectives in Plant Cell and Tissue Culture* provide a discussion of the basic techniques, problems, and potentials of plant tissue culture. There is ample evidence in all the contributions here that plant tissue culture technique is not an end in itself, as is sometimes alleged, but that it is a vigorous and living science, which has greatly improved, and will continue to improve, our understanding of plant biology. It has an important role to play in the future improvement and utilization of plants by man. During recent years there has been too much, and perhaps premature, emphasis on the applied and commercial aspects of plant tissue culture. For this reason, discussions in these two volumes are devoted to basic science, with relevant but only brief references to current and possible future applications.

Responsibility for the selection of various subjects that are discussed in these companion volumes, and their authors, is mine, and the choices clearly reflect my biases. All contributors have honored my request to work within the prescribed guidelines. However, treatment of the subject matter, and its interpretation, are the sole responsibility of the respective authors, and naturally reflect their own biases. Every attempt has been made to present a complete discussion of all of the issues and problems involved. It is never possible to maintain a uniform style of language and presentation in a contributed volume of this kind. I have, therefore, attempted to maintain the typical flavor of each contribution, which maintains the individual style and prose of each author. There is a small amount of unavoidable, and probably useful, overlap between some of the chapters, but that too serves to indicate the interdependence and interrelationships of the topics of discussion.

As one of those who appreciates the history of science, I believe it is as important for a student or research scientist to be aware of the most current information in his or her chosen field of research, as it is to be familiar with the evolution and development of research ideas. Too often these days only the current status of a field of research is discussed and valued, as though it were the whole and the final truth, and had no past. We fail to benefit from the efforts of countless scientists of earlier years who have played significant and often decisive roles in the development of modern ideas. A study of the history of science shows that scientific discoveries must always be treated as theories in development, for it is not too uncommon for today's dogmas to become the discredited or modified theories of tomorrow. The generation of scientific ideas and theories, and their

constant reevaluation and modification based on new findings, is one of the greatest pleasures of our profession. This we must share with the scientists of tomorrow. It is for this reason that most of the chapters also provide a historical perspective.

The meticulous care taken by all the contributors in the preparation of their chapters, and their adherence to time schedules, have made my editorial responsibilities lighter and a very pleasant experience. For these, I am grateful and thankful to all of my distinguished colleagues, many of whom had to write in an unfamiliar language. For me, personally, it has been a uniquely useful learning experience, and I hope that it will be the same for the readers of these volumes.

INDRA K. VASIL

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Chapter 1

# Cell Proliferation and Growth in Callus Cultures

M. M. YEOMAN AND E. FORCHE[1]

*Department of Botany, University of Edinburgh, Edinburgh, Scotland*



## I. Induction and Establishment of Callus

Wounding alone will induce proliferation at the damaged surface of an intact plant (Fig. 1) and may result in the formation of a callus (Yeoman, 1970). Such a callus usually persists for only a short time and rapidly becomes infiltrated with polyphenolic substances, which seal off the wound from the environment (Yeoman *et al.*, 1968). In order to sustain cell proliferation it is necessary to remove the developing callus and place it in culture in the presence of growth-promoting substances. The technical difficulty of securing and maintaining strict asepsis while removing and culturing a callus from an intact plant has led to the development of techniques for the induction and establishment of callus cultures. Here the initial inoculum is a tissue fragment, free from contaminating microorganisms, or a seedling from a sterile seed. Although mechanical wounding always accompanies explantation it is not a necessary prerequisite for callus initiation, which may be achieved directly from intact seedlings placed

[1] Present address: GBF D-3300 Braunschweig, Federal Republic of Germany.




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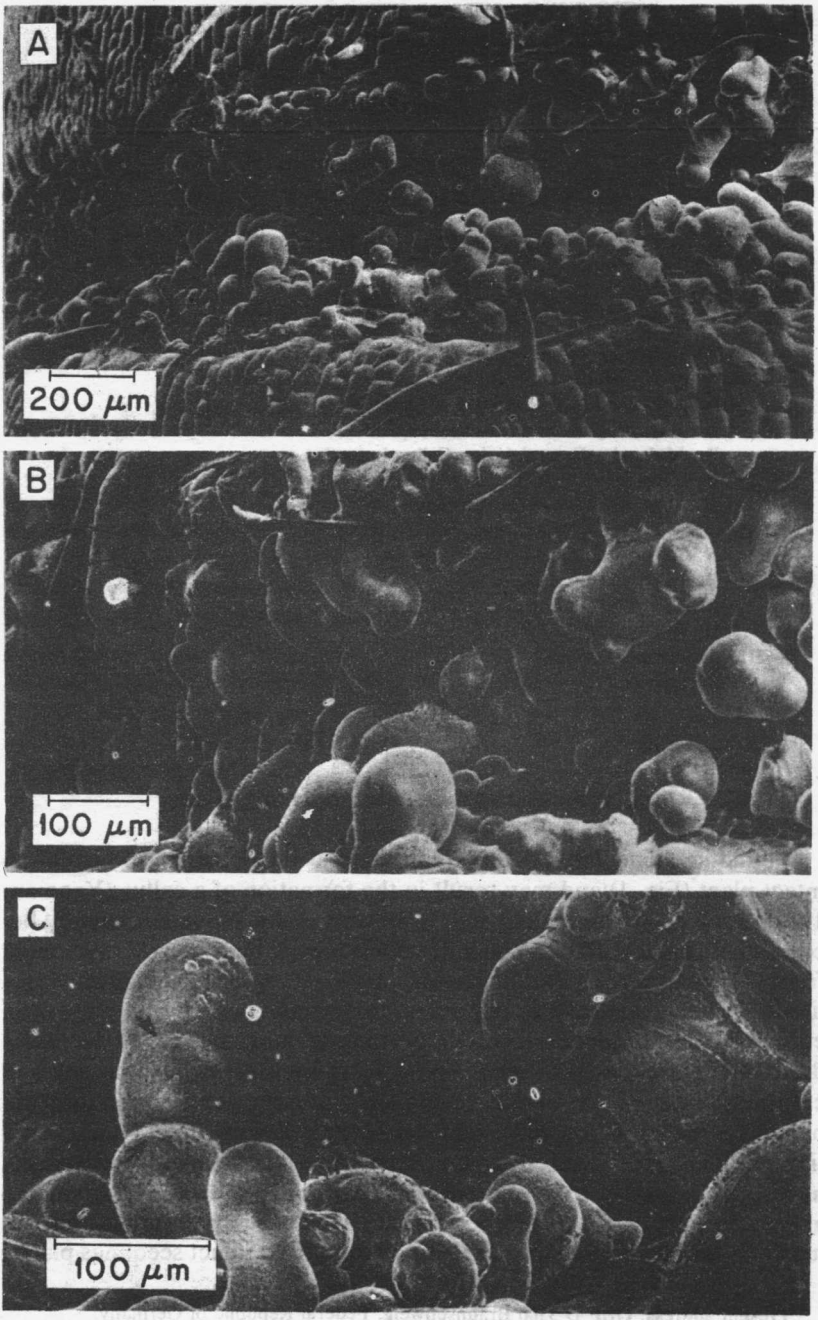
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in contact with growth-producing chemicals. From this it would appear that a marked change in the chemical environment of the constituent cells promoted by wounding and/or the addition of growth stimulants is the overriding factor in the initiation and establishment of a callus. Such agents modify or overcome two major regulatory controls within the tissue explant or intact plant: (1) the overall chemical environment of the tissue, provided by the long-distance transport of substances from other parts of the plant, and (2) the local molecular conversations that take place between adjacent cells. The release of wound substances and/or the effect of added chemical stimulants tend to obliterate the chemical environment of the cells before perturbation and to attentuate or overrule local conversations between cells, leading to a breakdown in cellular organization. Usually, the intact plant quickly reasserts control over the affected region and order is restored. Tissue explants and separated cells, however, are at the mercy of the chemical environment provided by the culture medium and the restoration of order is more difficult. Indeed, the maintenance of disorder depends on the continuous supply of chemicals that swamp the chemical regime within the explant and interfere with the molecular conversations between cells. Tissue systems maintained and subcultured in a disorganized state for extended periods of time by the provision of growth-promoting substances may eventually become independent of these substances and lose the ability to differentiate. Such cultures behave in a broadly similar fashion to tissue isolated from plant tumors. A permanent change in the cells, as with crown-gall tissue, depends on a seemingly irreversible change in metabolic status coupled with a marked decrease in the extent of communication between adjacent cells. Conversely, the emergence of organized structures from apparently disordered callus, which may be habituated, or cell cultures results from divisions in which the products remain in close contact, enabling an intimate local conversation or exchange of substances to become established and maintained. Subsequent regulated divisions lead to the formation of organized structures. In this Chapter attention is focused on the factors that affect the proliferative behavior of explanted tissues and callus in culture.

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FIG. 1. Scanning electron micrographs of callus formation in developing grafts. The grafts were 3 days old and the fresh material was photographed inside a Cambridge Cryostage at ca. −80°C. (A) Low-power magnification, showing the extensive development of callus cells along both surfaces of a heterograft between *Datura stramonium* and *Nicandra physaloides*. (B) The variety of shapes and disposition of the large callus cells can be clearly seen (plants as in A). (C) High-power magnification of a *Lycopersicon esculentum* homograft highlighting the shape and sequential divisions in a large callus cell (arrowed).

## A. Physiological Status of the Explant

The response of an explanted tissue to experimental treatments designed to induce callus depends on the physiological state of the tissue at the time of excision. Snijman *et al.* (1977) have shown that some of the variability of growth in pith explants removed from the stem of tabacco and placed in culture may be related to endogenous hormonal levels that change with distance from the terminal bud. Apart from the endogenous hormone balance, which clearly influences the ability of a tissue fragment to form callus, an important factor is the presence of inhibitory molecules, which may show seasonal variations. Katterman *et al.* (1977) have demonstrated that the addition of antioxidants (e.g., dithiothreitol) and auxin protectors (e.g., ascorbic acid) together with very high auxin levels (15–50 mg/liter of NAA) are conducive to the formation and viability of callus from seedlings of cotton (*Gossypium barbadense*). Similar observations have been reported by Green *et al.* (1974) working with *Zea mays*. Activated charcoal may be used to remove toxic substances emanating from anthers (Bajaj *et al.*, 1976; Wernicke and Kohlenbach, 1976), explants of date palm (Reuveni and Lilien-Kipnis, 1974), and in orchid culture (Wang and Huang, 1976). Care must be exercised in the use of activated charcoal, which can adsorb, in addition to growth inhibitors, a variety of plant growth hormones, effectively removing these from the culture medium (Weatherhead *et al.*, 1978).

Although some callus may be formed on explants from a variety of plants throughout the year, the intensity of the response tends to be related to the time of year when the tissue is removed. For example, the proportion of *Populus alba* explants forming callus increased markedly in March and was still high in November (Blanarikova and Karacsonyi, 1978). A systematic study of the metabolic changes that take place during the storage of Jerusalem artichoke tubers has been made by Robertson (1966) and Macleod *et al.* (1979). Robertson (1966) showed that explants removed from stored tubers at any time of the year would respond to conditions that promoted callus formation and that the subsequent rate of growth after 21 days in culture was similar despite considerable and progressive changes in the composition of the tissue. However, seasonal variation was observed in the time taken for the component cells of the explant to divide after excision and culture. This "lag phase" before the first division, which is synchronous (Yeoman and Evans, 1967) and involves up to 60% of the constituent cells (Yeoman and Davidson, 1971), remains relatively constant at 20–24 hours during the first 5 months of storage (ca. December–April) and then increases sharply, eventually, after 1 year in storage, reaching a time of approximately 60 hours. This lengthening of the lag phase is associated with a rundown of metabolic

potential and a gradual diminution of stored reserve metabolites. Inevitably these changes must reflect variations in the pattern of gene expression, which result in changes in the quality and quantity of RNA being transcribed at any particular time. Macleod *et al.* (1979) have shown that in Jerusalem artichoke the total amount of RNA per explant declined in a linear fashion from 8 to less than 5 μg over a period of 7 months (see Fig. 2). The contrast between the linear decrease in RNA and the nonlinear increase in time of the first wave of synchronous division suggests that a simple explanation relating the timing of the first wave of division to the amount of RNA in the explants and thence to the activity of the machinery for gene transcription is not tenable. However, the de-

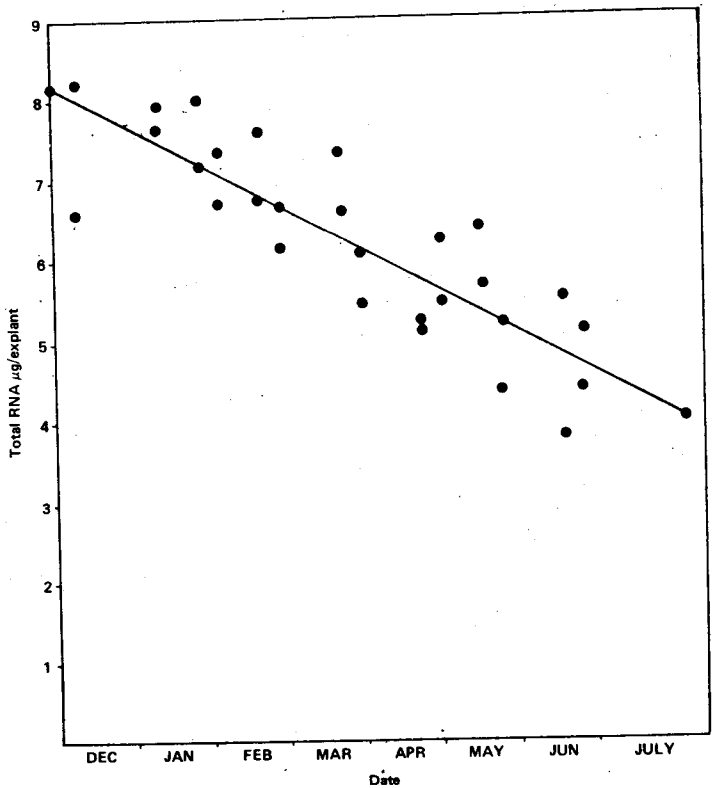


FIG. 2. The relationship between total RNA per freshly excised explant and the duration of tuber storage. The material used was excised from tubers of Jerusalem artichoke (*Helianthus tuberosus*) harvested in early December and stored in the dark at 4°C. The calculated regression line has a correlation coefficient of 81%.

cline in total RNA is so pronounced that a contribution to the overall effect from this source cannot be discounted.

Macleod *et al.* (1979) also recognized two distinct components in the overall response of Jerusalem artichoke tissue to culture in a callus-inducing medium. A wound response is exhibited when explants are cultured in a medium without auxin and does not induce callus formation, and a growth response is initiated by auxin and exemplified by the wave of cell division that commences after a short delay. The wound response, characterized by an early peak in the rate of uptake and incorporation of added precursors, seems to be much less susceptible to seasonal variations than the auxin-initiated growth-related responses, as seen in the timing of the onset of cell division. These seasonal variations appear to arise not from any change in the metabolic events leading up to cell division, but from a selective, continuous loss of enzyme activity in the stored tissue as the season progresses. The loss is presumably selective because the wound response shows less seasonal variation than the growth response. This stability may result either from intracellular compartmentation or from the development of a very stable set of enzymes necessary to cope with defense and repair of the tissue after wounding.

Many of the problems of inducing callus from plant tissues may be overcome by using intact, freshly germinated seedlings or explanted parts of these seedlings. This ensures that the tissue fragment is composed of cells with a high growth potential, thus circumventing the difficulties associated with the physiological status of the explants.

### B. Molecular Events during Callus Induction

Excision and culture promote considerable metabolic changes in plant cells (see Table I). Many of these changes in metabolism are reflected in alterations to the structure of cells that are preparing for division (Yeoman and Street, 1978). The appearance and shape of the nucleus changes, dense aggregations of chromatin are more frequently observed, and the frequency and appearance of mitochondria may alter (Bagshaw *et al.*, 1969). The appearance of the nucleolus changes (Vasil, 1973), the fibrillar and granular regions become intermingled, and electron-transparent regions may appear, around which ribosomal particles become clustered. This, taken together with an increase in the frequency of both free and attached ribosomes (Israel and Steward, 1966) in the cytoplasm and the appearance of polyribosomes, clearly informs us of the synthesis of the machinery for sustained protein synthesis. Storage products characteristic of the quiescent cells of the explant tend to disappear during the preparation for division. Starch vanishes from carrot cells brought into culture

TABLE I

CHANGES IN SELECTED GROWTH AND BIOCHEMICAL PARAMETERS DURING THE DEVELOPMENT OF A CALLUS FROM EXPLANTS OF JERUSALEM ARTICHOKE (*Helianthus tuberosus*)[a,b]

| Day | Fresh weight (mg) | Dry weight (mg) | Total nitrogen (μg N) | Cell number (× $10^3$) | Tracheid number (× $10^3$) | $CO_2$ (μl/hour/explant) | $O_2$ (μl/hour/explant) | RQ | DNP sensitivity (% $O_2$) | Phosphatase activity ($P_i$/hour/explant) |
|---|---|---|---|---|---|---|---|---|---|---|
| 0 | 8.4 | 1.1 | 43 | 23 | — | 0.4 | 1.4 | 0.3 | 5 | 1.5 |
| 1 | 8.5 | 0.9 | 37 | 22 | — | — | — | — | — | 1.3 |
| 2 | 9.5 | 1.2 | 18 | 30 | — | 2.8 | 3.3 | 0.8 | 11 | 3.9 |
| 3 | 10.5 | 1.5 | 32 | 58 | — | 3.6 | 4.2 | 0.9 | 13 | 2.4 |
| 4 | 12.7 | 1.4 | 53 | 133 | — | 5.1 | 5.3 | 1.0 | 19 | 3.7 |
| 5 | 13.8 | — | 83 | 222 | 5 | 6.9 | 6.2 | 1.1 | 48 | 4.7 |
| 6 | 16.6 | 1.4 | 120 | 288 | 12 | 7.6 | 6.4 | 1.2 | 5 | 4.8 |
| 7 | 17.8 | 1.8 | 128 | 488 | 18 | 8.8 | 7.4 | 1.2 | 4 | 5.6 |
| 10 | 21.5 | 2.4 | 193 | 600 | 50 | 9.0 | 7.6 | 1.2 | 16 | 7.1 |

[a] From Robertson (1966).

[b] All values have been expressed on a per explant basis.