

Plant Propagation by Tissue Culture
3rd Edition

Plant Propagation by Tissue Culture 3rd Edition

Volume 1. The Background

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Preface

It is now more than twenty years since the first edition of this work appeared and nearly fifteen since the second. Whilst much of the information in those editions has stood the test of time, inevitably, because of the pace of research, a new edition is clearly timely.

This is true, not only because many more species have been the subject of propagation studies, but because the background to the field – with which this volume deals – has changed almost out of all recognition. In particular, our knowledge of plant development, genetics physiology, biochemistry and molecular biology has expanded exponentially – often through work on mutants of *Arabidopsis* – and opened up many new avenues for the plant propagator to explore. Equally, the commercial significance of plant propagation has increased significantly. As an example, in the second edition there was a single chapter on plant growth regulators – in this there are three, reflecting the fact that not only is there more information on those PGRs we recognised in 1993, but that several new ones are now known. Equally, fifteen years ago we knew little of the molecular basis of plant development e.g. flower and shoot development, in this edition it has merited a whole chapter, much of which relates to discoveries in the last decade.

Because of these factors, it was felt that a different approach was required for this edition. The second edition was researched and written by Edwin George alone but it would now be very difficult for a single author to gain the breadth of expertise necessary to cover all the relevant aspects of this many-faceted subject. Hence, it was decided to adopt a multi-author approach, with chapters written by experts in their fields. These build upon the sound framework of the previous editions (which those with a knowledge of the previous works will recognise). Many sections of the previous work have been retained, but inevitably, apart from up-to-date reference lists, the text has undergone major revision in many areas.

Like the previous edition, the current one will appear in two volumes, but coverage has been extended and the order in which subjects are covered has been changed. Therefore, some topics, previously covered in Part 1, will now be discussed in Part 2. The ethos of the work is, as before, to produce an encyclopaedic text.

The first initiative to begin the new revision of *Plant Propagation by Tissue Culture* was made by Prof. A.C. Cassells and the editors are grateful to him for his early leadership. No work of this size can be accomplished successfully without much goodwill and hard work by the contributors, and to them the editors express their deepest thanks. We also express our sincere thanks to all those who have allowed us to use their material in diagrams and illustrations. We are very appreciative of the hard work by Dr. Susan Rafferty-McArdle of University College Cork in formatting the text, and to Dr. Jacco Flipsen of Springer for his support.

**Edwin George
Mike Hall
Geert-Jan de Klerk
May 2007**

Biographical Notes on Contributors

Chapter 1.

Edwin F. George trained as a botanist at Imperial College, London and subsequently gained a PhD, working on breeding and selection of sugar cane at the Mauritius Sugar Industry Research Institute. He was later employed by ICI Ltd. and Plant Protection Ltd. to study plant growth regulating compounds and subjects for corporate research. He finally became an independent consultant and researched extensively into plant genetic engineering and especially plant tissue culture. This resulted in the books *Plant Culture Media, Vols. 1 and 2* (1987), and *Plant Propagation by Tissue Culture*. The latter work was first published in 1984 and then extensively revised and extended to two volumes in 1993 and 1996. The present book is based on the first volume of the 2nd edition of *Plant Propagation by Tissue Culture*. Dr. George prepared the diagrams for the current revision although he is now retired.

Chapter 2.

Pierre C Debergh is Emeritus-Professor of the University of Gent (Belgium) since 2004 and specialised in micropropagation since 1968. His major interest is in tissue culture (*sensu largo*) and horticulture applied to western and developing countries (Asia, Africa and the Caribbean). He is editor of *Plant Cell Reports; Plant Cell, Tissue and Organ Culture* and the *South African Journal of Botany*. He is author of approx. 100 publications and supervisor of 35 PhD dissertations and more than 250 MSc dissertations.

Chapter 3.

Geert-Jan de Klerk is senior scientist in plant tissue culture since 1986, first in *The Centre for Plant Tissue Culture Research* in Lisse (Netherlands) and now in *Plant Research International, Wageningen University* (Netherlands). His main research interests concern plant developmental biology. He is editor-in-chief of *Plant Cell Tissue and Organ Culture* and editor of *Propagation of Ornamental Plants*.

Chapter 4.

Trevor A Thorpe was a PhD student of Toshio Murashige at the University of California, Riverside (USA). He was a Faculty Professor and now Professor Emeritus in the Department of Biological Sciences at the University of Calgary, Alberta, Canada. He retired in 1997 but is still an active

researcher. His areas of interest include developmental plant physiology, experimental plant morphogenesis and micropropagation, mainly of woody plants. He was a former Chairman of the International Association for Plant Tissue Culture and former editor-in-chief of *In Vitro Cellular and Developmental Biology – Plant*.

Edward C Yeung was a PhD student of I Sussex at Yale University. He is an Assistant Professor in the Department of Plant Science at the University of Manitoba (Canada). His research interests are structural, physiological and biochemical ontogeny of plant embryogenesis and floral biology of orchids

Claudio Stassolla was a PhD student of Edward Yeung at the University of Manitoba, (Canada). His research is on plant somatic embryogenesis *in vitro*.

Andy V Roberts is Emeritus Professor in the School of Health and Biosciences at the University of East London (UK). His research interests are the use of *in vitro* methods for the propagation and genetic improvement of woody plants, particularly roses.

Geert-Jan de Klerk (see chapter 3)

Chapter 5.

Ivana Machackova is a Professor at the Institute of Experimental Botany of the Academy of Sciences of the Czech Republic in Prague (Czech Republic). She is Head of the Laboratory of Plant Morphogenesis and Director of the Institute. She lectures in the Department of Plant Physiology at the Charles University in Prague. Her research interests are in the area of plant growth substances (auxins, ethylene, abscisic acid and melatonin); their modes of action and metabolism, regulation of their levels in relation to plant development and electrophysiology.

Eva Zazimalova is an Associate Professor of Plant Physiology at the Institute of Experimental Botany of the Academy of Sciences of the Czech Republic in Prague. She is Head of the Laboratory of Hormonal Regulation in Plants and Deputy Director of the Institute. She also teaches in the Department of Plant Physiology at the Charles University in Prague. Her research is in the fields of auxin and cytokinins (mode of action of auxin, auxin binding site(s), regulation of levels of auxins and cytokinins in relation to cell division and elongation and the mechanism of polar transport of auxin).

Chapter 6.

Johannes van Staden was awarded his PhD (Botany) in 1970 and lectured in this field until 2003. He is a Professor and Director of the Research Institute for Plant Growth and Development, School of Biological and Conservation Sciences, University of KwaZulu-Natal (South Africa). His main interests are in the hormonal regulation of plant growth, seed germination, plant tissue culture and ethnobotany/medicine.

Eva Zazimalova (see chapter 5).

Chapter 7.

Igor E Moshkov is a Leading Researcher in plant physiology and biochemistry and Deputy Director at the Timiryazev Institute of Plant Physiology, Russian Academy of Science, Moscow. His research is focussed on ethylene signal perception and transduction, the interaction between ethylene and cytokinin at the level of hormone perception and signal transduction pathways and GTP-binding proteins in phytohormone signalling.

Galina V Novikova is a Leading Researcher in plant physiology and biochemistry at the Timiryazev Institute of Plant Physiology, Russian Academy of Science, Moscow. Her research is related to the mode of action of phytohormone action (cytokinins and ethylene) and interactions of the phytohormones, protein phosphorylation/dephosphorylation in relation to phytohormone signal perception and transduction and MAPK cascades in phytohormone signal transduction.

Michael A Hall has been Professor of Botany at the University of Wales, Aberystwyth (UK) since 1981. His research is involved with signal perception and transduction mechanisms for plant hormones, especially ethylene, as well as the role of hormones in the responses of plants to environmental stress.

Chapter 8.

Dominique Chriqui is Professor and Director of a laboratory of plant development at the University Pierre and Marie Curie, Paris (France). She has been involved for many years in research on the cellular and molecular features that underlie morphogenic events such as rhizogenesis and shoot regeneration, both in planta and *in vitro*. She is now particularly interested in the early events of the regenerative process and in the interfaces between hormones, cell cycle and developmental genes and has published approx. 100 papers in the field of plant morphogenesis.

Chapter 9.

Sara von Arnold holds a PhD from Uppsala University (1979), Sweden. She has been a full Professor in the Cell Biology of forest trees at the Swedish University of Agricultural Sciences, Uppsala since 1988. Her research focusses on developmental processes in conifers and especially somatic embryogenesis.

Chapter 10.

Peter B Gahan is Emeritus Professor of Cell Biology at King's College London (UK) with fifty years of research and teaching experience in plant and animal biology. He is interested in the mechanism of competence and recalcitrance of plant cells to regenerate and also in the role of DNA as a messenger between cells and tissues.

Chapter 11.

John Preece is a horticulture professor in the Department of Plant, Soil and Agricultural Systems at Southern Illinois University Carbondale (USA). He teaches courses in General Horticulture, Plant Propagation and Plant Growth and Development. He conducts research on various aspects of woody plant propagation. Along with his postgraduates, he was the first to publish micropropagation protocols for a number of woody species and the first to work out somatic embryogenesis and shoot morphogenesis of *Fraxinus americana* (white ash) and *Juglans nigra* (eastern black walnut).

Chapter 12.

William (Bill) Davies is currently Professor of Environmental Plant Biology at Lancaster University (UK) and Director of the Lancaster Environmental centre, one of the largest groups of environmental researchers in Europe. He obtained his first degree in Horticultural Science from the University of Reading (UK) and his PhD in Forestry and Botany from the University of Wisconsin, Madison (USA). His research interests include regulation of growth and functioning of plants experiencing environmental stress; stomatal physiology, root to shoot communication via chemical signalling in plants; environmental physiology of crops and native species; crop improvement for water-scarce environments; irrigation science and enhancing the efficiency of crop water use through novel management techniques. He has published more than 200 papers in international plant science journals and edited 17 books. He is a member of the ISI database of 'Highly Cited Researchers' in Plant and Animal Sciences. He is a member of the Defra Horticulture

Link programme Management Committee and editor-in-chief of the *Journal of Experimental Botany*.

Chapter 13.

Meira Ziv is a Professor in the Robert H Smith Institute of Plant Science and Genetics at the Hebrew University of Jerusalem (Israel). Her research interests are in the physiology and morphogenesis of plant organogenesis and somatic embryogenesis in large scale liquid cultures; shoot-malformation, hyperhydricity and the role of oxidative stress in the

control of plant development in bioreactor cultures for efficient acclimatization and survival *ex vitro*; bulb and corm development in geophytes cultured in liquid cultures in relation to carbohydrate metabolism.

Jianxin Chen is a research scientist in the Department of Biology at Brock University, Ontario (Canada). His interests are in large-scale micropropagation, metabolic pathways and cloning of medicinal plants and plant breeding.

Chapter 1

Plant Tissue Culture Procedure - Background

1. INTRODUCTION

Plant tissue culture is the science of growing plant cells, tissues or organs isolated from the mother plant, on artificial media. It includes techniques and methods used to research into many botanical disciplines and has several practical objectives. Before beginning to propagate plants by tissue culture methods, it is necessary to have a clear understanding of the ways in which plant material can be grown and manipulated in 'test tubes'. This chapter therefore describes the techniques that have been developed for the isolation and *in vitro* culture of plant material, and shows where further information can be obtained. Both organised and unorganised growth are possible *in vitro*.

1.1. ORGANISED GROWTH

Organised growth contributes towards the creation or maintenance of a defined structure. It occurs when plant organs such as the growing points of shoots or roots (apical meristems), leaf initials, young flower buds or small fruits, are transferred to culture and continue to grow with their structure preserved. Growth that is coherently organised also occurs when organs are induced. This may occur *in vitro* either directly upon an organ or upon a piece of tissue placed in culture (an explant), or during the culture of previously unorganised tissues. The process of *de novo* organ formation is called

organogenesis or morphogenesis (the development of form).

1.2. UNORGANISED GROWTH

The growth of higher plants depends on the organised allocation of functions to organs which in consequence become differentiated, that is to say, modified and specialised to enable them undertake their essential roles. Unorganised growth is seldom found in nature, but occurs fairly frequently when pieces of whole plants are cultured *in vitro*. The cell aggregates, which are then formed, typically lack any recognisable structure and contain only a limited number of the many kinds of specialised and differentiated cells found in an intact plant. A differentiated cell is one that has developed a specialised form (morphology) and/or function (physiology). A differentiated tissue (e.g. xylem or epidermis) is an aggregation of differentiated cells. So far, the formation of differentiated cell types can only be controlled to a limited extent in culture. It is not possible, for example, to maintain and multiply a culture composed entirely of epidermal cells. By contrast, unorganised tissues can be increased in volume by subculture and can be maintained on semi-solid or liquid media for long periods. They can often also be used to commence cell suspension cultures. Differentiation is also used botanically to describe the formation of distinct organs through morphogenesis.

2. TISSUE CULTURE

2.1. CULTURES OF ORGANISED STRUCTURES

Organ culture is used as a general term for those types of culture in which an organised form of growth can be continuously maintained. It includes the aseptic isolation from whole plants of such definite structures as leaf primordia, immature flowers and fruits, and their growth *in vitro*. For the purposes of plant propagation, the most important kinds of organ culture are:

- Meristem cultures, in which are grown very small excised shoot apices, each consisting of the apical meristematic dome with or without one or two leaf primordia. The shoot apex is typically grown to give one single shoot.

- Shoot tip, or shoot cultures, started from excised shoot tips, or buds, larger than the shoot apices employed to establish meristem cultures, having several leaf primordia. These shoot apices are usually cultured in such a way that each produces multiple shoots.

- Node cultures of separate lateral buds, each carried on a small piece of stem tissue; stem pieces carrying either single or multiple nodes may be cultured. Each bud is grown to provide a single shoot.

- Isolated root cultures. The growth of roots, unconnected to shoots: a branched root system may be obtained.

- Embryo cultures, where fertilised or unfertilised zygotic (seed) embryos are dissected out of developing seeds or fruits and cultured *in vitro* until they have grown into seedlings. Embryo culture is quite distinct from somatic embryogenesis (see below).

These types of cultures are described in more detail later in this chapter.

2.2. CULTURES OF UNORGANISED TISSUES

‘Tissue culture’ is commonly used as a collective term to describe all kinds of *in vitro* plant cultures although strictly it should refer only to cultures of unorganised aggregates of cells. In practice the following kinds of cultures are most generally recognised:

- Callus (or tissue) cultures. The growth and maintenance of largely unorganised cell masses, which arise from the uncoordinated and disorganised growth of small plant organs, pieces of plant tissue, or previously cultured cells.
- Suspension (or cell) cultures. Populations of plant cells and small cell clumps, dispersed in an agitated, that is aerated, liquid medium.
- Protoplast cultures. The culture of plant cells that have been isolated without a cell wall.
- Anther cultures. The culture of complete anthers containing immature pollen microspores. The objective is usually to obtain haploid plants by the formation of somatic embryos (see below) directly from the pollen, or sometimes by organogenesis *via* callus. Pollen cultures are those initiated from pollen that has been removed from anthers.

2.3. USING TISSUE CULTURES FOR PLANT PROPAGATION

The objective of plant propagation via tissue culture, termed micropropagation, is to propagate plants true-to-type, that is, as clones. Plants obtained from tissue culture are called microplants and can be derived from tissue cultures in three ways:

- from pre-existing shoot buds or primordial buds (meristems) which are encouraged to grow and proliferate;
- following shoot morphogenesis when new shoots are induced to form in unorganised tissues or directly upon explanted tissues of the mother plant;
- through the formation of somatic embryos which resemble the seed embryos of intact plants, and which can grow into seedlings in the same way. This process is called somatic embryogenesis.

To obtain plants by the first two of these methods, it is necessary to treat shoots of an adequate size as miniature cuttings and induce them to produce roots.

The derivation of new plants from cells, which would not normally have taken part in the process of regeneration, shows that living, differentiated plant cells may express totipotency, i.e. they each retain a latent capacity to produce a whole plant. Totipotency is a special characteristic of cells in young tissues and meristems. It can be exhibited by some differentiated cells, e.g. cambial cells and leaf palisade cells but not those which have developed into terminally differentiated structures (e.g. sieve tubes or tracheids).

Theoretically, plant cells, organs, or plants, can all be cloned, i.e., produced in large numbers as a population where all the individuals have the same genetic constitution as the parent. Present tissue culture techniques do not permit this in every case and irregularities do sometimes occur, resulting in ‘somaclonal variants’ (Larkin and Scowcroft, 1981). Nevertheless, as will be described in the chapters, which follow, a very large measure of success can be achieved and cultures of various kinds can be used to propagate plants.

2.4. INITIATING TISSUE CULTURES

2.4.1. Explants

Tissue cultures are started from pieces of whole plants. The small organs or pieces of tissue that are used are called explants. The part of the plant (the stock plant or mother plant) from which explants are obtained, depends on:

- the kind of culture to be initiated;
- the purpose of the proposed culture;
- the plant species to be used.

Explants can therefore be of many different kinds. The correct choice of explant material can have an important effect on the success of tissue culture. Plants growing in the external environment are invariably contaminated with micro-organisms and pests. These contaminants are mainly confined to the outer surfaces of the plant, although, some microbes and viruses may be systemic within the tissues (Cassels, 1997). Because they are started from small explants and must be grown on nutritive media that are also favourable for the growth of micro-organisms, plant tissue cultures must usually be established and maintained in aseptic conditions. Most kinds of microbial organism, and in particular bacteria and fungi, compete adversely with plant material growing *in vitro*. Therefore, as far as

possible, explants must be free from microbial contaminants when they are first placed on a nutrient medium. This usually involves growing stock plants in ways that will minimise infection, treating the plant material with disinfecting chemicals to kill superficial microbes, and sterilising the tools used for dissection and the vessels and media in which cultures are grown (for a review see Cassells and Doyle, 2005). Some kinds of plants can, however, be micropropagated in non-sterile environments (see Chapter 3).

2.4.2. Isolation and incubation

The work of isolating and transferring cultured plant material is usually performed in special rooms or inside hoods or cabinets from which micro-organisms can be excluded. Cabinets used for isolation can be placed in a draught-free part of a general laboratory, but are much better situated in a special inoculation or transfer room reserved for the purpose. The accommodation, equipment and methods that are required for successful inoculation and transfer are described in Volume 2. Cultures, once initiated, are placed in incubators or growth rooms where lighting, temperature and humidity can be controlled. The rate of growth of a culture will depend on the temperature (and sometimes the lighting) regime adopted.

2.4.3. The cultural environment

Plant cultures are commenced by placing one or more explants into a pre-sterilised container of sterile nutrient medium. Some explants may fail to grow, or may die, due to microbial contamination: to ensure the survival of an adequate number, it therefore is usual to initiate several cultures at the same time, each being started from an identical organ or piece of tissue. Explants taken from stock plants at different times of the year may not give reproducible results in tissue culture. This may be due to variation in the level of external contaminants or because of seasonal changes in endogenous (internal) growth regulator levels in the stock plant (see Chapter 11).

2.4.4. Media

Plant material will only grow *in vitro* when provided with specialised media. A medium usually consists of a solution of salts supplying the major and minor elements necessary for the growth of whole plants, together with:

- various vitamins (optional);
- various amino acids (optional);

- an energy source (usually sucrose).

The components of plant tissue culture media are discussed in Chapters 3 and 4. The compositions of specific media are described in Volume 2. Growth and development of plant cultures usually also depends on the addition of plant growth regulators to the medium (see Chapters 5, 6 and 7). Plant growth regulators are compounds, which, at very low concentration, are capable of modifying growth or plant morphogenesis. Many workers define a medium as a completed mixture of nutrients and growth regulators. This is a rather inflexible method, as growth regulators frequently need to be altered according to the variety of plant, or at different stages of culture, whilst the basic medium can stay unchanged. It is therefore recommended that nutritional and regulatory components should be listed separately. Plant material can be cultured either in a liquid medium or on a medium that has been partially solidified with a gelling agent (see Chapter 4). The method employed will depend on the type of culture and its objective.

2.4.5. Solidified media

Media which have had a gelling agent added to them, so that they have become semi-solid, are widely used for explant establishment; they are also employed for much routine culture of callus or plant organs (including micropropagation), and for the long-term maintenance of cultures. Agar is the most common solidifying agent, but a gellan gum is also widely used (Chapter 4).

Cultures grown on solid media are kept static. They require only simple containers of glass or plastic, which occupy little space. Only the lower surface of the explant, organ or tissue is in contact with the medium. This means that as growth proceeds there may be gradients in nutrients, growth factors and the waste products of metabolism, between the medium and the tissues. Gaseous diffusion into and out of the cells at the base of the organ or tissue may also be restricted by the surrounding medium.

2.4.6. Liquid media

Liquid media are essential for suspension cultures, and are preferred for critical experiments on the nutrition, growth and cell differentiation in callus tissues. They are also used in some micropropagation work. Very small organs (e.g. anthers) are often floated on the top of liquid medium and plant cells or protoplasts can be cultured in very shallow layers of

static liquid, providing there is sufficient gaseous diffusion. Larger organs such as shoots (e.g. proliferating shoots of shoot cultures) can also often be grown satisfactorily in a shallow layer of non-agitated liquid where part of the organ protrudes above the surface. However, some method of support is necessary for small organs or small pieces of tissue, which would otherwise sink below the surface of a static liquid medium, or they will die for lack of aeration. Systems of support which have been found to be effective and which can be used instead of agar-solidified media are described in Chapters 4.

Many tissues and organs, small and large, also grow well unsupported in a liquid medium, providing it is aerated by shaking or moving (see below). Some kind of agitation is essential for suspension cultures to prevent cells and cell aggregates settling to the bottom of the flask. Other purposes served by agitation include: the provision of increased aeration, the reduction of plant polarity, the uniform distribution of nutrients and the dilution of toxic explant exudates (Lim-Ho, 1982).

There are several alternative techniques. Plant cell suspensions can be cultured very satisfactorily when totally immersed in a liquid culture medium, providing it is shaken (by a rotary or reciprocating shaking machine) or stirred (e.g. by a magnetic stirrer) to ensure adequate aeration. This method may also be used for culturing organs of some plants (e.g.

proliferating shoot cultures), but the fragmentation, which occurs, can be disadvantageous.

Periodic immersion may be achieved by growing cultured material in tubes or flasks of liquid medium which are rotated slowly. Steward and Shantz (1956) devised so-called 'nipple flasks' for this purpose which had several side-arms. They were fixed to a wooden wheel, which was rotated so that tissue in the arms of each flask was alternately bathed in medium and drained or exposed to the air (Fig. 1.1). This technique ensured that callus tissue for which they were used was well aerated. The medium usually became turbid as cells dissociated from the callus and started a cell suspension. Flasks of this sort are seldom used to-day because of their cost. A similar alternating exposure can be achieved by placing calluses in vessels, which are rotated slowly.

An alternative to the costly rotating systems to achieve periodic immersion of the cultures, is the increasingly popular temporary immersion system in which static vessels are periodically or temporarily flooded with culture medium (Fig. 1.2; Teisson and Alvard, 1995). Medium is pumped from a reservoir container into the culture vessel for experimentally determined time intervals repeated over a 24 hour cycle. This system prevents anoxia and has the advantage that the medium can easily be changed in the reservoir.

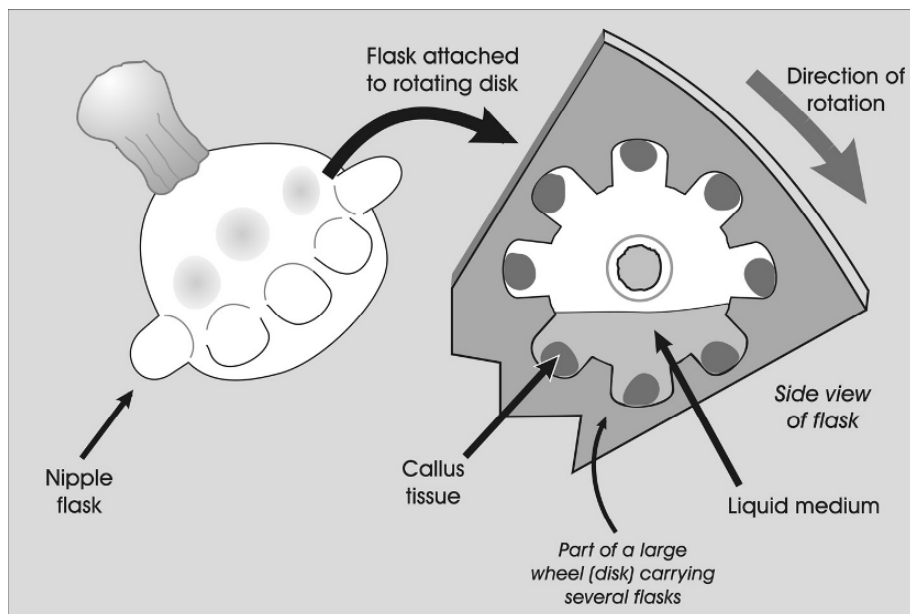


Fig. 1.1 A nipple flask for growing callus in a liquid medium.

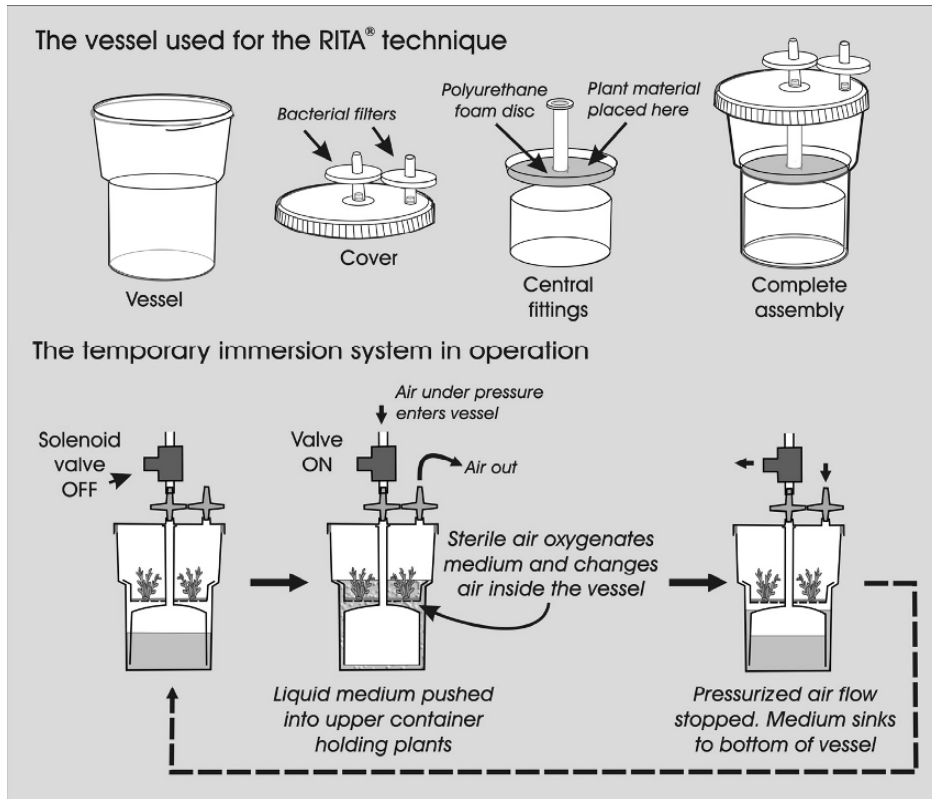


Fig. 1.2 An illustration of the RITA ebb and flow culture vessel.

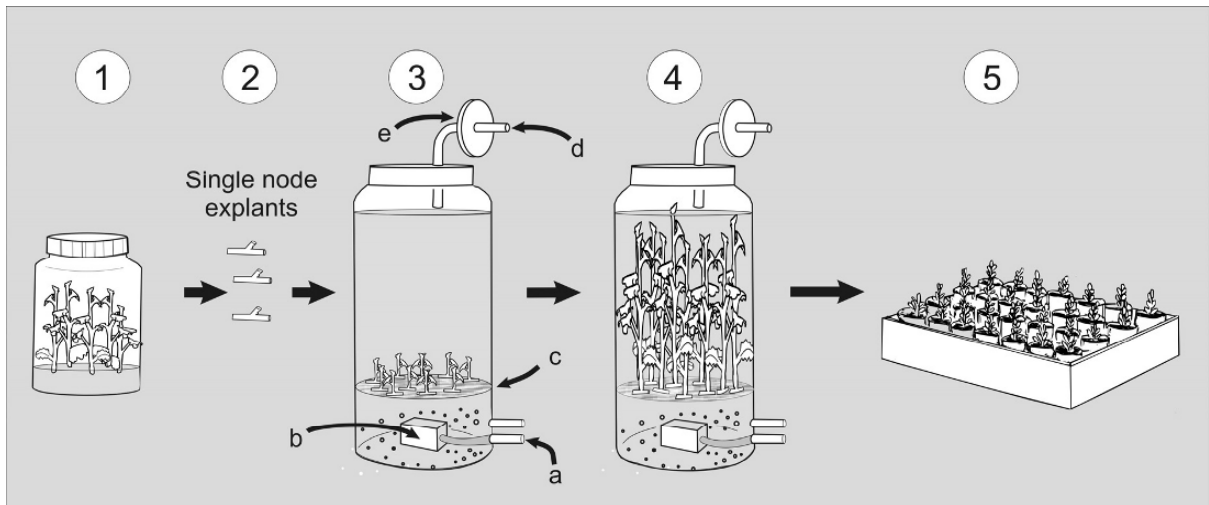


Fig. 1.3 An illustration of micropropagation in a bioreactor.

a. air inlet; b. sparger; c. raft supporting explants; d. air outlet; e. membrane filter. The bioreactor culture is initiated by inoculation with nodes or buds from conventional agar culture. For details of bioreactor design see Fig. 1.9.

Liquid medium in flasks or column bio-reactors (fermentors) can be circulated and at the same time aerated, by the introduction of sterile air. Shearing

forces within air-lift reactors are much less than in mechanically-stirred vessels so that plant cell suspensions suffer less damage. Bio-reactors are

used in the pharmaceutical industry to produce high value plant secondary products and to carry out substrate conversions. Low cost bio-reactors developed for micropropagation have been described in detail in Hvorslef-Eide and Preil (2005) (Fig. 1.3).

Rather than immersing callus or organ cultures, liquid medium may be slowly dripped onto the growing tissues or applied as a mist and afterwards the liquid drained or pumped away for recirculation (Weathers and Giles, 1987). A particular advantage of this technique is the ability to grow cultures in a constant and non-depleted medium; nutrients can be varied frequently and rapidly and their availability controlled by altering either concentration or flow rate. Toxic metabolites, which in a closed container might accumulate and inhibit growth, can be removed continuously. As complicated apparatus is needed, the method has not been widely used.

The relative merits of solid and liquid media (and combinations of both) are discussed further in Chapter 12.

2.5. PROBLEMS OF ESTABLISHMENT

2.5.1. Phenolic oxidation

Some plants, particularly tropical species, contain high concentrations of phenolic substances that are oxidised when cells are wounded or senescent. Isolated tissue then becomes brown or black and fails to grow. The prevention of blackening, which can be a serious problem in tissue culture, is discussed in Chapter 11.

2.5.2. Minimum inoculation density

Certain essential substances can pass out of plant cells by diffusion. Substances known to be released into the medium by this means include alkaloids, amino acids, enzymes, growth substances and vitamins (Street, 1969). The loss is of no consequence when there is a large cluster of cells growing in close proximity or where the ratio of plant material to medium is high. However, when cells are inoculated onto an ordinary growth medium at a low population density, the concentration of essential substances in the cells and in the medium can become inadequate for the survival of the culture. For successful culture initiation, there is thus a minimum size of explant or quantity of separated cells or protoplasts per unit culture volume. Inoculation density also affects the initial rate of growth *in vitro*. Large explants generally survive more frequently and grow more rapidly at the outset than very small ones. In practice, minimum inoculation density varies

according to the genotype of plant being cultured and the cultural conditions. For commencing suspension cultures it is commonly about $1-1.5 \times 10^4$ cells/ml.

The minimum cell density phenomenon is sometimes called a 'feeder effect' because deficiencies can often be made up by the presence of other cells growing nearby. Suspension cultures can be started from a low density of inoculum by 'conditioning' a freshly prepared medium - i.e. allowing products to diffuse into it from a medium in which another culture is growing actively, or adding a quantity of filter-sterilised medium which has previously supported another culture. The use of conditioned media can reduce the critical initial cell density by a factor of about 10 (Stuart and Street, 1969).

It is possible to overcome the deficiencies of plant cells at low starting densities by adding small amounts of known chemicals to a medium. For example, Kao and Michayluk (1975) have shown that *Vicia hajastana* cells or protoplasts can be cultured from very small initial inocula or even from individual cells: a standard culture medium was supplemented with growth regulators, several organic acids, additional sugars (apart from sucrose and glucose), and in particular, casein hydrolysate (casamino acids) and coconut milk.

There is often a maximum as well as a minimum plating or inoculation density for plant cells or protoplasts. In a few cases the effective range has been found to be quite narrow. Some effects of inoculation density on morphogenesis are described in Chapter 10.

2.6. PATTERNS OF GROWTH AND DIFFERENTIATION

A typical unorganised plant callus, initiated from a new explant or a piece of a previously-established culture, has three stages of development, namely:

- the induction of cell division;
- a period of active cell division during which differentiated cells lose any specialised features they may have acquired and become dedifferentiated;
- a period when cell division slows down or ceases and when, within the callus, there is increasing cellular differentiation.

These phases are similarly reproduced by cell suspensions grown in a finite volume of medium (a batch culture), where according to a variety of different parameters that can be used to measure growth (e.g. cell number, cell dry weight, total DNA

content) an S-shaped growth curve is generally obtained (Fig. 1.4).

The phases are:

- a lag phase;
- a period of exponential and then linear growth;
- a period when the rate of growth declines;
- a stationary phase when growth comes to a halt.

Some differentiation of cells may occur in cell cultures during the period of slowed and stationary

growth, but generally it is less marked and less complete than that which occurs in callus cultures. Cultures cannot be maintained in stationary phase for long periods. Cells begin to die and, as their contents enter the nutrient medium, death of the whole culture accelerates. Somewhat similar patterns of growth also occur in cultures of organised structures. These also cease growth and become moribund as the components of the medium become exhausted.

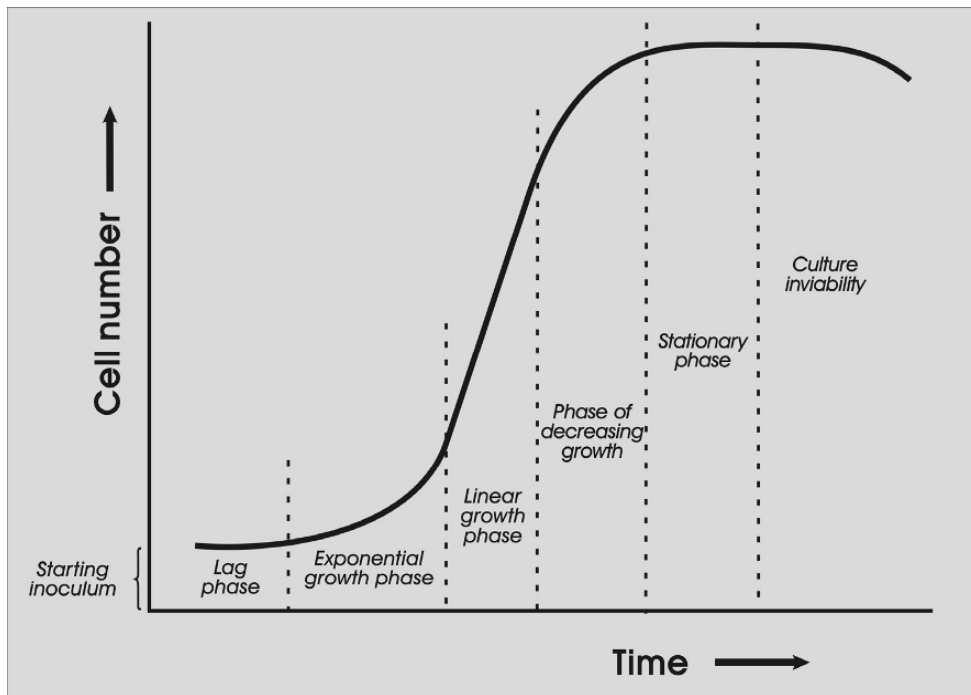


Fig. 1.4 Diagram showing the phases of growth in batch suspension culture.

2.7. SUBCULTURING

Once a particular kind of organised or unorganised growth has been started *in vitro*, it will usually continue if callus cultures, suspension cultures, or cultures of indeterminate organs (see below) are divided to provide new explants for culture initiation on fresh medium. Subculturing often becomes imperative when the density of cells, tissue or organs becomes excessive; to increase the volume of a culture; or to increase the number of organs (e.g. shoots or somatic embryos) for micropropagation. The period from the initiation of a culture or a subculture to the time of its transfer is sometimes called a passage. The first passage is that in which the original explant or inoculum is introduced.

Suspensions regularly subcultured at the end of the period of exponential growth can often be propagated over many passages. However, many cultures reach a peak of cell aggregation at this time and aggregation often becomes progressively more pronounced in subsequent passages (Street, 1977b). Subculture is therefore more conveniently carried out during the stationary phase when cell aggregation is least pronounced. Rapid rates of plant propagation depend on the ability to subculture shoots from proliferating shoot or node cultures, from cultures giving direct shoot regeneration, or callus or suspensions capable of reliable shoot or embryo regeneration.

A further reason for transfer, or subculture, is that the growth of plant material in a closed vessel eventually leads to the accumulation of toxic metabolites and the exhaustion of the medium, or to its drying out. Thus, even to maintain the culture, all or part of it must be transferred onto fresh medium. Callus subcultures are usually initiated by moving a fragment of the initial callus (an inoculum) to fresh medium in another vessel. Shoot cultures are subcultured by segmenting individual shoots or shoot clusters. The interval between subcultures depends on the rate at which a culture has grown: at 25°C, subculturing is typically required every 4-6 weeks. In the early stages of callus growth it may be convenient to transfer the whole piece of tissue to fresh medium, but a more established culture will need to be divided and only small selected portions used as inocula. Regrowth depends on the transfer of healthy tissues.

Decontamination procedures are theoretically no longer necessary during subculturing, although sterile transfer procedures must still be used. However, when using shoot or node cultures for micropropagation, some laboratories do re-sterilise plant material at this stage as a precaution against the spread of contaminants (see Volume 2). Cultures which are obviously infected with micro-organisms should not be used for subculturing and should be autoclaved before disposal.

3. TYPES OF TISSUE CULTURE

3.1. ORGAN CULTURES

Differentiated plant organs can usually be grown in culture without loss of integrity. They can be of two types:

- Determinate organs which are destined to have only a defined size and shape (e.g. leaves, flowers and fruits);
- Indeterminate organs, where growth is potentially unlimited (apical meristems of roots and non-flowering shoots).

In the past, it has been thought that the meristematic cells within root or shoot apices were not committed to a particular kind of development. It is now accepted that, like the primordia of determinate organs such as leaves, apical meristems also become inherently programmed (or determined) into either root or shoot pathways (see Chapter 8). The eventual pattern of development of both indeterminate and determinate organs is often established at a very early stage. For example, the meristematic protrusions in a shoot apex become

2.8. SUBCULTURING HAZARDS

There are several hazards in subculturing which are discussed more fully in other chapters of this book. Several kinds of callus may arise from the initial explant, each with different morphogenic potential. Strains of callus tissue capable of giving rise to somatic embryos and others without this capability can, for instance, arise simultaneously from the culture of grass and cereal seed embryos. Careful selection of the correct strain is therefore necessary if cultures capable of producing somatic embryos are ultimately required. Timing of the transfer may also be important, because if left alone for some while, non-embryogenic callus may grow from the original explant at the expense of the competent tissue, which will then be obscured or lost.

Although subculturing can often be continued over many months without adverse effects becoming apparent, cultures of most unorganised cells and of some organised structures can accumulate cells that are genetically changed. This may cause the characteristics of the culture to be altered and may mean that some of the plants regenerated from the culture will not be the same as the parent plant. This subject is discussed further in Chapter 2. Cultures may also inexplicably decline in vigour after a number of passages, so that further subculture becomes impossible.

programmed to develop as either lateral buds or leaves after only a few cell divisions have taken place (see Chapter 10).

3.1.1. Culture of determinate organs

An organ arises from a group of meristematic cells. In an indeterminate organ, such cells are theoretically able to continue in the same pattern of growth indefinitely. The situation is different in the primordium of a determinate organ. Here, as meristematic cells receive instructions on how to differentiate, their capacity for further division becomes limited.

If the primordium of a determinate organ is excised and transferred to culture, it will sometimes continue to grow to maturity. The organ obtained *in vitro* may be smaller than that which would have developed on the original plant *in vivo*, but otherwise is likely to be normal. The growth of determinate organs cannot be extended by subculture as growth ceases when they have reached their maximum size.

Organs of limited growth potential, which have been cultured, include leaves (Caponetti and Steeves, 1963; Caponetti, 1972); fruits (Nitsch, 1951, 1963; Street, 1969); stamens (Rastogi and Sawhney, 1988); ovaries and ovules (which develop and grow into embryos) and flower buds of several dicotyledonous plant species (Table 1.1).

Until recently, a completely normal development was obtained in only a few cases. This was probably due to the use of media of sub-optimum composition. By experimenting with media constituents, Berghoef and Bruinsma (1979a) obtained normal growth of *Begonia franconis* buds and were thus able to study the effect of plant growth substances and nutritional factors on flower development and sexual expression (Berghoef and Bruinsma, 1979b). Similarly, by culturing dormant buds of *Salix*, Angrish and Nanda (1982a,b) could study the effect of bud position and the progressive influence of a resting period on the determination of meristems to become catkins and fertile flowers. In several species, flowers have been pollinated *in vitro* and have then given rise to mature fruits (e.g. Ruddat *et al.*, 1979)

Table 1.1 Some species in which flower buds have been cultured

<i>Cucumis sativus</i>	Galun <i>et al.</i> (1962)
<i>Viscaria spp.</i>	Blake (1966, 1969)
<i>Nicotiana tabacum</i>	Hicks and Sussex (1970)
<i>Aquilegia formosa</i>	Bilderback (1971)
<i>Cleome iberidella</i>	De Jong and Bruinsma (1974)
<i>Nicotiana offinis</i>	Deaton <i>et al.</i> (1980)

Plants cannot be propagated by culturing meristems already committed to produce determinate organs, but providing development has not proceeded too far, flower meristems can often be induced to revert to vegetative meristems *in vitro*. In some plants the production of vegetative shoots from the flower meristems on a large inflorescence can provide a convenient method of micropropagation (see Chapter 2).

3.1.2. Culture of indeterminate organs

Meristem and shoot culture. The growing points of shoots can be cultured in such a way that they continue uninterrupted and organised growth. As these shoot initials ultimately give rise to small organised shoots which can then be rooted, their culture has great practical significance for plant propagation. Two important uses have emerged:

Meristem culture. Culture of the extreme tip of the shoot, is used as a technique to free plants from virus infections. Explants are dissected from either apical or lateral buds. They comprise a very small stem apex (0.2-1.0 mm in length) consisting of just the apical meristem and one or two leaf primordia;

Shoot culture or shoot tip culture. Culture of larger stem apices or lateral buds (ranging from 5 or 10 mm in length to undissected buds) is used as a very successful method of propagating plants.

The size and relative positions of the two kinds of explant in a shoot apex of a typical dicotyledon is shown in Fig. 1.5. Node culture is an adaptation of shoot culture.

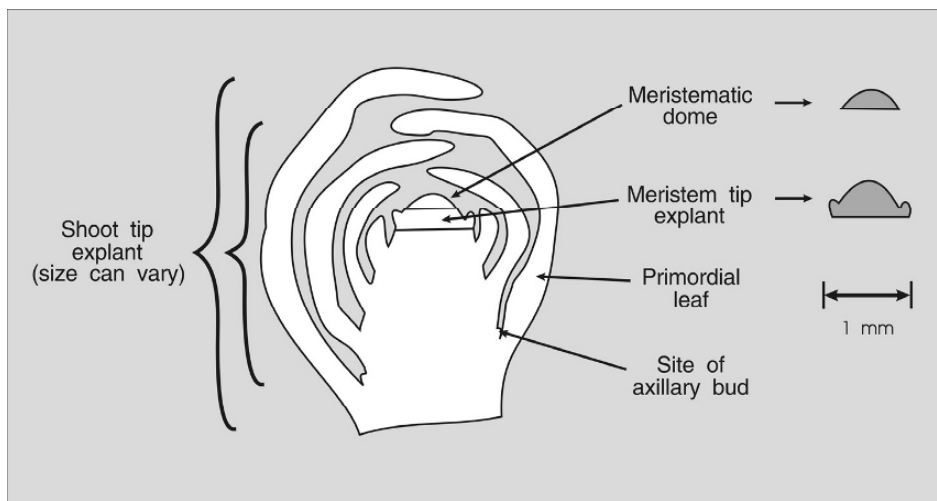


Fig. 1.5 A diagrammatic section through a bud showing the locations and approximate relative sizes of a meristematic dome, the meristem tip and shoot tip explants.

If successful, meristem culture, shoot culture and node culture can ultimately result in the growth of small shoots. With appropriate treatments, these original shoots can either be rooted to produce small plants or 'plantlets', or their axillary buds can be induced to grow to form a cluster of shoots. Plants are propagated by dividing and reculturing the shoot clusters, or by growing individual shoots for subdivision. At a chosen stage, individual shoots or shoot clusters are rooted. Tissue cultured shoots are removed from aseptic conditions at or just before the rooting stage, and rooted plantlets are hardened off and grown normally. Shoot culture, node culture and meristem tip culture are discussed in greater detail in Chapter 2.

Embryo culture. Zygotic or seed embryos are often used advantageously as explants in plant tissue culture, for example, to initiate callus cultures. In embryo culture however, embryos are dissected from seeds, individually isolated and 'germinated' *in vitro* to provide one plant per explant. Isolated embryo culture can assist in the rapid production of seedlings from seeds that have a protracted dormancy period, and it enables seedlings to be produced when the genotype (e.g. that resulting from some interspecific crosses) conveys a low embryo or seed viability.

During the course of evolution, natural incompatibility systems have developed which limit the types of possible sexual crosses (see De Nettancourt and Devreux, 1977). Two kinds of infertility occur:

- Pre-zygotic incompatibility, preventing pollen germination and/or pollen tube growth so that a zygote is never formed;
- Post-zygotic incompatibility, in which a zygote is produced but not accepted by the endosperm. The embryo, not receiving sufficient nutrition, disintegrates or aborts.

Pre-zygotic incompatibility can sometimes be overcome in the laboratory using a technique developed by Kanta *et al.* (1962) called *in vitro* pollination (or *in vitro* fertilisation). For a description of this technique see review articles by Ranga Swamy (1977), Zenkteler (1980) and Yeung *et al.* (1981). Reviews of embryo culture have been provided by Torrey (1973), Norstog (1979) and Raghavan (1967, 1977a, 1980).

Embryo culture has been used successfully in a large number of plant genera to overcome post-zygotic incompatibility which otherwise hampers the production of desirable hybrid seedlings. For

example, in trying to transfer insect resistance from a wild *Solanum* species into the aubergine, Sharma *et al.* (1980a) obtained a few hybrid plants (*Solanum melongena* x *S. khasianum*) by embryo culture. Embryo culture in these circumstances is more aptly termed embryo rescue. Success rates are usually quite low and the new hybrids, particularly if they arise from remote crosses, are sometimes sterile. However, this does not matter if the plants can afterwards be propagated asexually. Hybrids between incompatible varieties of tree and soft fruits (Tukey, 1934; Skirm, 1942) and *Iris* (in Reuther, 1977) have been obtained by culturing fairly mature embryos.

Fruits or seeds are surface sterilised before embryo removal. Providing aseptic techniques are strictly adhered to during excision and transfer to a culture medium, the embryo itself needs no further sterilisation. To ease the dissection of the embryo, hard seeds are soaked in water to soften them, but if softening takes more than a few hours it is advisable to re-sterilise the seed afterwards. A dissecting microscope may be necessary to excise the embryos from small seeds as it is particularly important that the embryo should not be damaged.

Culture of immature embryos (pro-embryos) a few days after pollination frequently results in a greater proportion of seedlings being obtained than if more mature embryos are used as explants, because incompatibility mechanisms have less time to take effect. Unfortunately dissection of very small embryos requires much skill and cannot be done rapidly: it also frequently results in damage which prevents growth *in vitro*. In soybean, Hu and Sussex (1986) obtained the best *in vitro* growth of immature embryos if they were isolated with their suspensors intact. Excised embryos usually develop into seedlings precociously (i.e. before they have reached the size they would have attained in a normal seed).

As an alternative to embryo culture, in some plants it has been possible to excise and culture pollinated ovaries and immature ovules. Ovule culture, sometimes called '*in ovulo* embryo culture', can be more successful than the culture of young embryos. Pro-embryos generally require a complex medium for growth, but embryos contained within the ovule require less complicated media. They are also easily removed from the plant and relatively insensitive to the physical conditions of culture (Thengane *et al.*, 1986). The difference

between embryo and ovule culture is shown diagrammatically in Fig. 1.6.

Because seedlings, which resulted from ovule culture of a *Nicotiana* interspecific cross all died after they had developed some true leaves, Iwai *et al.* (1985) used leaves of the immature seedlings as explants for the initiation of callus cultures. Most shoots regenerated from the callus also died at an early stage, but one gave rise to a plant, which was discovered later to be a sterile hybrid. Plants were also regenerated from callus of a *Pelargonium* hybrid by Kato and Tokumasu (1983). The callus in this case arose directly from globular or heart-shaped zygotic embryos which were not able to grow into seedlings.

The seeds of orchids have neither functional storage organs, nor a true seed coat, so dissection of the embryo would not be possible. In fact, for commercial purposes, orchid seeds are now almost always germinated *in vitro*, and growth is often facilitated by taking immature seeds from green pods (see Volume 2).

Many media have been especially developed for embryo culture and some were the forerunners of the media now used for general tissue culture. Commonly, mature embryos require only inorganic salts supplemented with sucrose, whereas immature embryos have an additional requirement for vitamins, amino acids, growth regulators and sometimes coconut milk or some other endosperm extract. Raghavan (1977b) encouraged the incorporation of mannitol to replace the high osmotic pressure exerted on proembryos by ovular sap. Seedlings obtained from embryos grown *in vitro* are planted out and hardened off in the same manner as other plantlets raised by tissue culture (Chapter 2 and Volume 2).

Although embryo culture is especially useful for plant breeders, it does not lead to the rapid and large scale rates of propagation characteristic of other micropropagation techniques, and so it is not considered further in this book. More details can be found in papers by: Sanders and Ziebur (1958); Raghavan (1967, 1980); Torrey (1973); Zilis and Meyer (1976); Collins and Grosser (1984), Monnier (1990) and Ramming (1990). Yeung *et al.* (1981) have suggested a basic protocol, which with modifications, should be applicable to any species.

The induction of multiple shoots from seeds is described in Chapter 2.

Isolated root culture. Root cultures can be established from root tips taken from primary or lateral roots of many plants. Suitable explants are

small sections of roots bearing a primary or lateral root meristem. These explants may be obtained, for example, from surface sterilised seeds germinated in aseptic conditions. If the small root meristems continue normal growth on a suitable medium, they produce a root system consisting only of primary and lateral roots (Fig. 1.7.). No organised shoot buds will be formed.

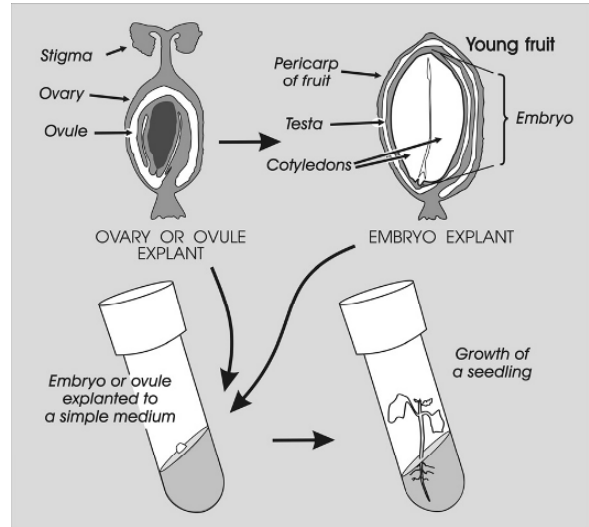


Fig. 1.6 Ovule and embryo culture.

The discovery that roots could be grown apart from shoot tissue was one of the first significant developments of modern tissue culture science. Root culture initially attracted a great deal of attention from research workers and the roots of many different species of plants were cultured successfully (see the comprehensive reviews of Street, 1954, 1957, 1969; and Butcher and Street, 1964).

Plants fall generally into three categories with regard to the ease with which their roots can be cultured. There are some species such as clover, *Datura*, tomato and *Citrus*, where isolated roots can be grown for long periods of time, some seemingly, indefinitely (Said and Murashige, 1979) providing regular subcultures are made. In many woody species, roots have not been grown at all successfully in isolated cultures. In other species such as pea, flax and wheat, roots can be cultured for long periods but ultimately growth declines or insufficient lateral roots are produced to provide explants for subculture.

The inability to maintain isolated root cultures is due to an induced meristematic dormancy or 'senescence', related to the length of time that the

roots have been growing *in vitro*. Transferring dormant meristems to fresh medium does not promote regrowth, possibly due to the accumulation of naturally-occurring auxinic growth substances at the root apex. The addition of so-called anti-auxin, or cytokinin growth regulators can often prolong active

growth of root cultures, whereas placing auxins or gibberellic acid in the growth medium, causes it to cease more rapidly. Cultures, which cannot be maintained by transferring root apices, can sometimes be continued if newly-initiated lateral root meristems are used as secondary explants instead.

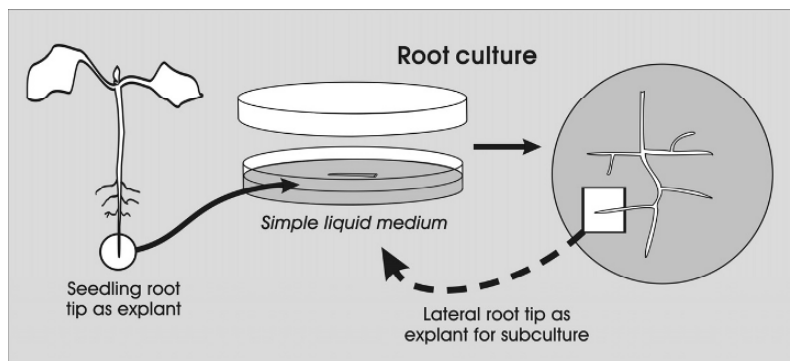


Fig. 1.7 Methods of root culture.

Isolated plant roots can usually be cultured on relatively simple media such as White (1954) containing 2% sucrose. Liquid media are preferable, as growth in or on a solid medium is slower. This is presumably because salts are less readily available to the roots from a solidified medium and oxygen availability may be restricted. Although roots will accept a mixed nitrate/ammonium source, they will not usually grow on ammonium nitrogen alone. Species, and even varieties or strains, of plants, are found to differ in their requirement for growth regulators, particularly for auxins, in the root culture medium.

Isolated root cultures have been employed for a number of different research purposes. They have been particularly valuable in the study of nematode infections and provide a method by which these parasites can be cultured in aseptic conditions. Root cultures may also be used to grow beneficial mycorrhizal fungi, and to study the process of root nodulation with nitrogen-fixing *Rhizobium* bacteria in leguminous plants. For the latter purpose, various special adaptations of standard techniques have been adopted to allow roots to become established in a nitrate-free medium (Raggio *et al.*, 1957; Torrey, 1963).

Unlike some other cultured tissues, root cultures exhibit a high degree of genetic stability (see Chapter 10). It has therefore been suggested that root cultures could afford one means of storing the germplasm of certain species (see Volume 2). For suitable species,

root cultures can provide a convenient source of explant material for the micropropagation of plants, but they will only be useful in micropropagation if shoots can be regenerated from roots. There are however, several ways in which this can be done, although they are likely to be effective in only a small number of plant genera which have a natural tendency to produce suckers, or new shoots from whole or severed roots:

- From direct adventitious shoots;
- From shoots or embryos originating indirectly on root callus;
- By conversion of the apical root meristem to a shoot meristem.

Adventitious shoots form readily on the severed roots of some plant species, and root cuttings are employed by horticulturists to increase plants *in vivo* (see, for example, the review by Hodge, 1986). Shoot regeneration from roots has not been widely used as a method of micropropagation, even though direct shoot regeneration from roots has been observed *in vitro* on many plants. Sections of fleshy roots used as primary explants are especially likely to form new shoots. Adventitious shoots always develop at the proximal end of a root section while, as a rule, new roots are produced from the distal end. Isolated root cultures would be useful in micropropagation if shoots could be induced to form directly upon them. Unfortunately plants seem to have a high degree of genetic specificity in their

capacity to produce shoots directly on isolated root cultures. Shoot induction often occurs after the addition of a cytokinin to the medium. Seeliger (1956) obtained shoot buds on cultured roots of *Robinia pseudoacacia* and Torrey (1958), shoot buds on root cultures of *Convolvulus*. Direct shoot formation was induced in three species of *Nicotiana* and on *Solanum melongena* by Zelcer *et al.* (1983) but in *N. tabacum* and *N. petunoides* shoots were only obtained after callus formed on the roots. The most optimistic report we have seen comes from Mudge *et al.* (1986), who thought that the shoot formation, which they could induce in raspberry root cultures would provide a convenient and labour-saving method of multiplying this plant *in vitro*.

Plants may also be regenerated from root-derived callus of some species e.g. tomato (Norton and Boll, 1954); *Isatis tinctoria* (Danckwardt-Lilliestrom, 1957); *Atropa belladonna* (Thomas and Street, 1972). Embryogenesis, leading to the formation of protocorm-like bodies, occurs in the callus derived from the root tips of certain orchids e.g. *Catasetum trulla* x *Catasetum* (Kerbaux, 1984a); *Epidendrum obrienianum* (Stewart and Button, 1978); *Oncidium varicosum* (Kerbaux, 1984b).

Changing the determined nature of a root meristem, so that it is induced to produce a shoot instead of a root, is a very rare event but has been noted to occur *in vitro* in the orchid *Vanilla planifolia*. The quiescent centre of cultured root tip meristems was changed into a shoot meristem so that cultured root tips grew to produce plantlets or multiple shoots (Philip and Nainar, 1986). Ballade (1971) maintained that newly initiated root initials, arising from single nodes of *Nasturtium officinale*, could be made to develop into shoot meristems by placing a crystal of kinetin on each explant which was then transferred to a medium containing 0.05% glucose.

3.2. CULTURE OF UNORGANISED CELLS

3.2.1. Callus cultures

Callus is a coherent and amorphous tissue, formed when plant cells multiply in a disorganised way. It is often induced in or upon parts of an intact plant by wounding, by the presence of insects or micro-organisms, or as a result of stress. Callus can be initiated *in vitro* by placing small pieces of the whole plant (explants) onto a growth-supporting medium under sterile conditions. Under the stimulus of endogenous growth regulators or growth regulating chemicals added to the medium, the metabolism of

cells, which were in a quiescent state, is changed, and they begin active division. During this process, cell differentiation and specialisation, which may have been occurring in the intact plant, are reversed, and the explant gives rise to new tissue, which is composed of meristematic and unspecialised cell types.

During dedifferentiation, storage products typically found in resting cells tend to disappear. New meristems are formed in the tissue and these give rise to undifferentiated parenchymatous cells without any of the structural order that was characteristic of the organ or tissue from which they were derived. Although callus remains unorganised, as growth proceeds, some kinds of specialised cells may again be formed. Such differentiation can appear to take place at random, but may be associated with centres of morphogenesis, which can give rise to organs such as roots, shoots and embryos. The *de novo* production of plants from unorganised cultures is often referred to as plant regeneration.

Although most experiments have been conducted with the tissues of higher plants, callus cultures can be established from gymnosperms, ferns, mosses and thallophytes. Many parts of a whole plant may have an ultimate potential to proliferate *in vitro*, but it is frequently found that callus cultures are more easily established from some organs than others. Young meristematic tissues are most suitable, but meristematic areas in older parts of a plant, such as the cambium, can give rise to callus. The choice of tissues from which cultures can be started is greatest in dicotyledonous species. A difference in the capacity of tissue to give rise to callus is particularly apparent in monocotyledons. In most cereals, for example, callus growth can only be obtained from organs such as zygotic embryos, germinating seeds, seed endosperm or the seedling mesocotyl, and very young leaves or leaf sheaths, but so far never from mature leaf tissue (e.g. Green and Phillips, 1975; Dunstan *et al.*, 1978). In sugar cane, callus cultures can only be started from young leaves or leaf bases, not from semi-mature or mature leaf blades.

Even closely associated tissues within one organ may have different potentials for callus origination. Thus when embryos of *Hordeum distichum* at an early stage of differentiation are removed from developing seeds and placed in culture, callus proliferation originates from meristematic mesocotyl cells rather than from the closely adjacent cells of the scutellum and coleorhiza (Granatek and Cockerline, 1979).

The callus formed on an original explant is called 'primary callus'. Secondary callus cultures are initiated from pieces of tissue dissected from primary callus (Fig. 1.8.). Subculture can then often be

continued over many years, but the longer callus is maintained, the greater is the risk that the cells thereof will suffer genetic change (see Chapter 10).

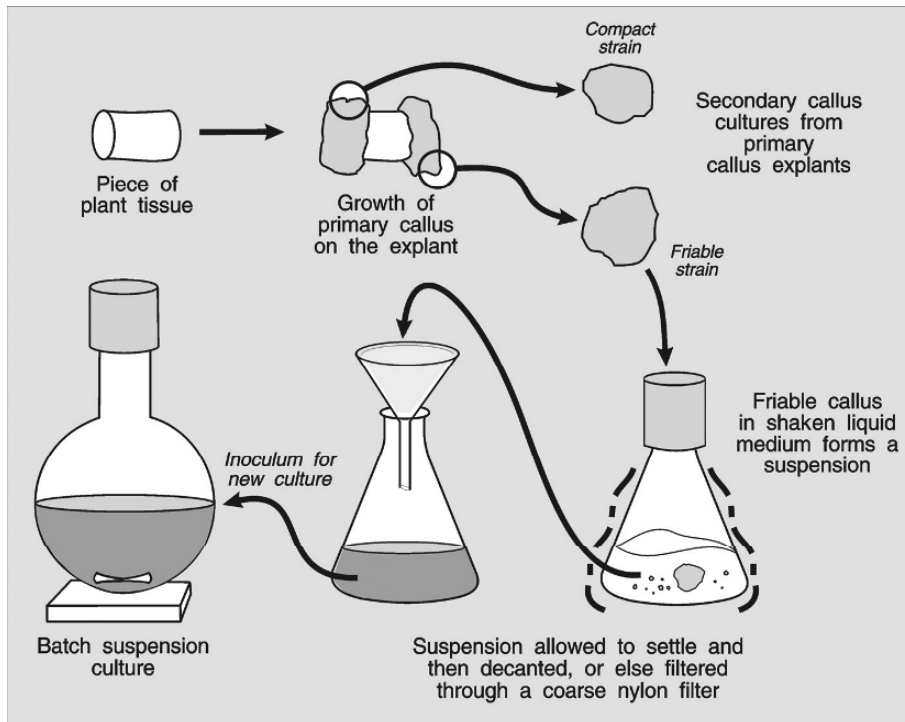


Fig. 1.8 Typical steps in the initiation of callus and suspension cultures.

Callus tissue is not of one single kind. Strains of callus differing in appearance, colour, degree of compaction and morphogenetic potential commonly arise from a single explant. Sometimes the type of callus obtained, its degree of cellular differentiation and its capacity to regenerate new plants, depend upon the origin and age of the tissue chosen as an explant. Loosely packed or 'friable' callus is usually selected for initiating suspension cultures (see below).

Some of the differences between one strain of callus tissue and another can depend on which genetic programme is functioning within the cells (epigenetic differences). Variability is more likely when callus is derived from an explant composed of more than one kind of cell. For this reason there is often merit in selecting small explants from only morphologically uniform tissue, bearing in mind that a minimum size of explant is normally required to obtain callus formation.

The genetic make up of cells is very commonly altered in unorganised callus and suspension cultures. Therefore another reason for cell strains having different characteristics, is that they have become composed of populations of cells with slightly different genotypes. Genetic and epigenetic changes occurring in cultures are described in greater detail in Chapters 10 and Volume 2. The growth, structure, organisation and cytology of callus are discussed in various chapters of the book edited by Street (1977a), and also in the review by Yeoman and Forche (1980).

3.2.2. Cell suspension cultures

Unorganised plant cells can be grown as callus in aggregated tissue masses, or they can be freely dispersed in agitated liquid media. Techniques are similar to those used for the large-scale culture of bacteria. Cell or suspension cultures, as they are called, are usually started by placing an inoculum of friable callus in a liquid medium (Fig. 1.8). Under agitation, single cells break off and, by division, form

cell chains and clumps which fracture again to give individual cells and other small cell groups. It is not always necessary to have a previous callus phase before initiating suspension cultures. For example, leaf sections of *Chenopodium rubrum* floated on Murashige and Skoog (1962) medium in the light, show rapid growth and cell division in the mesophyll, and after 4 days on a rotary shaker they can be disintegrated completely to release a great number of cells into suspension (Geile and Wagner, 1980).

Because the walls of plant cells have a natural tendency to adhere, it is not possible to obtain suspensions that consist only of dispersed single cells. Some progress has been made in selecting cell lines with increased cell separation, but cultures of completely isolated cells have yet to be obtained. The proportion and size of small cell aggregates varies according to plant variety and the medium in which the culture is grown. As cells tend to divide more frequently in aggregates than in isolation, the size of cell clusters increases during the phase of rapid cell division. Because agitation causes single cells, and small groups of cells, to be detached, the size of cell clusters decreases in batch cultures as they approach a stationary growth phase (see below).

The degree of cell dispersion in suspension cultures is particularly influenced by the concentration of growth regulators in the culture medium. Auxinic growth regulators increase the specific activity of enzymes, which bring about the dissolution of the middle lamella of plant cell walls (Torrey and Reinert, 1961). Thus by using a relatively high concentration of an auxin and a low concentration of a cytokinin growth regulator in the culture medium, it is usually possible to increase cell dispersion (Narayanawamy, 1977). However, the use of high auxin levels to obtain maximum cell dispersion will ensure that the cultured cells remain undifferentiated. This may be a disadvantage if a suspension is being used to produce secondary metabolites. Well-dispersed suspension cultures consist of thin-walled undifferentiated cells, but these are never uniform in size and shape. Cells with more differentiated structure, possessing, for example, thicker walls and even tracheid-like elements, usually only occur in large cell aggregates.

Many different methods of suspension culture have been developed. They fall into two main types: batch cultures in which cells are nurtured in a fixed volume of medium until growth ceases, and continuous cultures in which cell growth is maintained by continuous replenishment of sterile

nutrient media. All techniques utilise some method of agitating the culture medium to ensure necessary cell dispersion and an adequate gas exchange.

Batch cultures. Batch cultures are initiated by inoculating cells into a fixed volume of nutrient medium. As growth proceeds, the amount of cell material increases until nutrients in the medium are depleted or there is the accumulation of an inhibitory metabolite. Batch cultures have a number of disadvantages that restrict their suitability for extended studies of growth and metabolism, or for the industrial production of plant cells, but they are nevertheless widely used for many laboratory investigations. Small cultures are frequently agitated on orbital shakers onto which are fixed suitable containers, which range in volume from 100 ml (Erlenmeyer conical flasks) to 1000 ml (spherical flasks); the quantity of medium being approximately the same as the flask volume. The shakers are usually operated at speeds from 30-180 rpm with an orbital motion of about 3 cm. Alternatively, stirred systems can be used.

Continuous cultures. Using batch cultures, it is difficult to obtain a steady rate of production of new cells having constant size and composition. Attempts to do so necessitate frequent sub-culturing, at intervals equivalent to the doubling time of the cell population. Satisfactorily balanced growth can only be produced in continuous culture, a method, which is especially important when plant cells are to be used for the large-scale production of a primary or secondary metabolite. Continuous culture techniques require fairly complicated apparatus. Agitation of larger cultures in bio-reactors is usually achieved by stirring with a turbine and/or by passing sterile air (or a controlled gaseous mixture) into the culture from below and releasing it through plugged vents. Mechanically stirred reactors damage plant cells by shearing. This is minimised in air-lift reactors. Different bioreactor designs are illustrated in Fig. 1.9.

The use of suspension cultures in plant propagation. The growth of plant cells is more rapid in suspension than in callus culture and is also more readily controlled because the culture medium can be easily amended or changed. Organs can be induced to develop in cell suspensions: root and shoot initiation usually commences in cell aggregates. Somatic embryos may arise from single cells. Cells from suspensions can also be plated onto solid media where single cells and/or cell aggregates grow into callus colonies from which plants can often be regenerated. For these reasons suspension cultures

might be expected to provide a means of very rapid plant multiplication. There are two methods:

- plants may be obtained from somatic embryos formed in suspensions. Once embryos have been produced, they are normally grown into plantlets on solid media, although other methods are potentially available (Chapter 2);

- cells from suspensions are plated onto solid media where single cells and/or cell aggregates grow into callus colonies from which plants can often be regenerated.

In practice neither of these techniques has been sufficiently reliable for use in plant propagation.

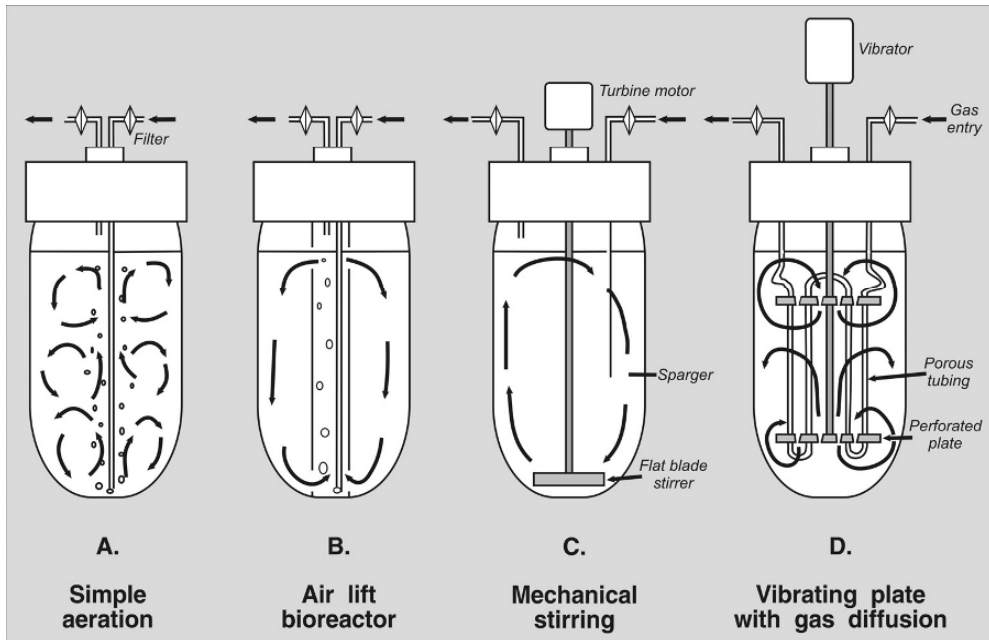


Fig.1.9 Four types of bioreactors used for plant cell culture.

Immobilised cell cultures. Plant cells can be captured and immobilised by being cultured in a gel which is afterwards solidified (see Chapter 4). This technique has only limited application to plant micropropagation, but is now employed quite widely when plant cells are grown for the production of their secondary products or for the bio-transformation of chemical compounds (Lindsey and Yeoman, 1983).

3.3. CULTURES OF SINGLE CELL ORIGIN

3.3.1. Single cell clones

Cultures can be initiated from single plant cells, but only when special techniques are employed. Frequently these comprise passing suspension-cultured cells through a filter which removes coarse cell aggregates and allows only single cells and very small cell clusters to pass through. Small groups of cells are then assumed to have originated from single cells. The suspension obtained is usually plated onto (or incorporated into) a solidified medium in Petri

dishes at a sufficient density to permit cell growth (see below), but with the cells sufficiently dispersed so that, when growth commences, individual callus colonies can be recognised under a binocular microscope and transferred separately to fresh medium. Cell lines originating from single cells in this way are sometimes called single cell clones or cell strains. The derivation of single cell clones was reviewed by Street (1977c).

Each cell clone has a minimum effective initial cell density (or minimum inoculation density) below which it cannot be cultured. The minimum density varies according to the medium and growth regulators in which the cells are placed; it is frequently about 10–15 cells/ml on standard media. Widely dispersed cells or protoplasts will not grow because they lose essential growth factors into the surrounding medium. The minimum inoculation density can therefore be lowered by adding to a standard medium either a filtered extract of a medium in which a culture has

been previously grown (the medium is then said to be conditioned), or special organic additives (when it is said to be supplemented).

Cells or protoplasts (see below) plated at a density which is insufficient for spontaneous cell division may also be nurtured into initial growth by being 'nursed' by tissue growing nearby. One way of doing this is to place an inoculum onto a filter paper disc

(a raft) or some other inert porous material, which is then put in contact with an established callus culture of a similar species of plant, the cells of which are called nurse cells, and the tissue a feeder layer. An alternative technique is to divide a Petri dish into compartments (Fig. 1.10.). Nurse tissues cultured in some segments assist the growth of cells or protoplasts plated in the other areas.

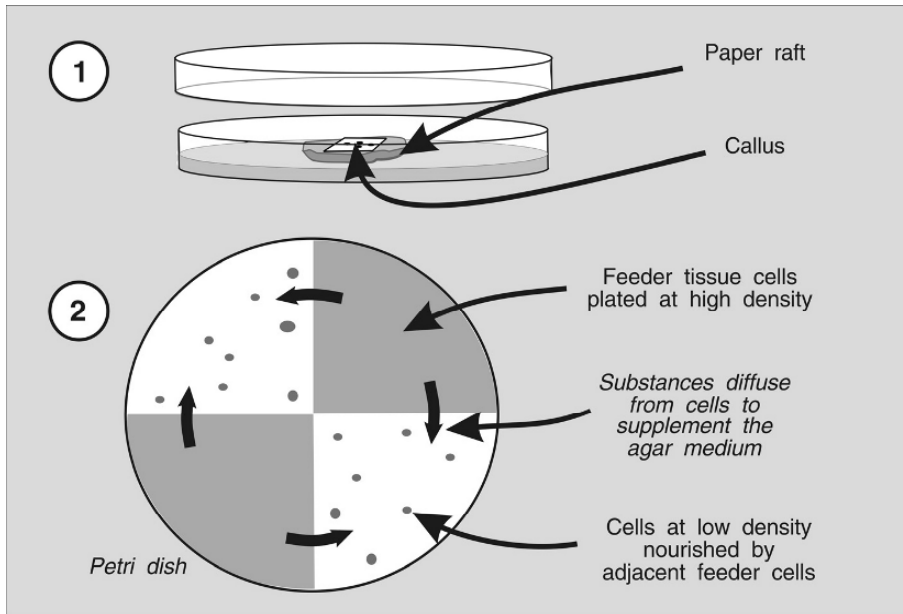


Fig. 1.10 Two methods of assisting the growth of cells plated at low density.

Another method of producing cell colonies which are very likely to have had a single cell origin, has been described by Bellincampi *et al.* (1985). A filtered cell suspension with a high proportion of single cells, is cultured at high density in a medium which contains only 0.2% agar. At this concentration the agar does not solidify the medium, but keeps apart the cell colonies growing from individual cells, preventing them from aggregating. When clusters of approximately 10-15 cells have been formed, they can be plated at a dilution of 50 (20% plating efficiency) to 200 plating units/ml (60% plating efficiency) on a medium gelled with 1% agar where they grow as separate callus colonies. Plating efficiency is the percentage of plating units (cell aggregates in this case) which give rise to callus colonies

The establishment of single cell clones is one way to separate genetically different cell lines from a mixed cell population. By artificially increasing the

genetic variation between cells in a culture, and then applying a specific selection pressure, resistant cell lines have been obtained (e.g. those resistant to certain drugs, herbicides or high levels of salt), and in some instances plants with similar resistances have then been regenerated from the resulting cells or callus (Dix, 1990).

3.3.2. Separated cells

Single cells can be separated directly from intact plants. They are often more easily isolated and less liable to damage than protoplasts, because the cell wall remains intact. Consequently, single cells can be used in robust operations, such as direct physiological studies. It has been said that, for this purpose, they are more representative of differentiated tissues than cells derived from tissue cultures (Miksch and Beiderbeck, 1976); but the disruption caused by separation may induce atypical responses.

Mechanical separation. In some plant species, disrupting the tissue mechanically can separate intact cells of certain organs. Viable mesophyll cells, for example, can be obtained easily from *Asparagus cladodes* (Colman *et al.*, 1979) and from leaves of *Macleaya cordata* (Kohlenbach; 1966, 1967). These cells can be grown either in suspension or solid culture and induced into morphogenesis, including somatic embryo formation (Kohlenbach, 1977). Schwenk (1980, 1981) simply placed pieces of the young cotyledons of sweet potato in water inside an abrasive tube in which a vortex was created. After removing debris, a cell suspension could be obtained from which cells grew and formed callus when plated on nutrient agar.

However, the capacity to isolate separated cells directly from higher plants appears to be limited (Jullien and Rossini, 1977). The type of tissue used seems to be important both to permit cell separation and to obtain subsequent growth. Cells separated from the leaves, instead of from the cotyledons, of sweet potato (above) had no capacity for growth, and it was not possible to even separate cells by mechanical means from several other plants.

Enzymatic separation. Cell separation can be assisted by treating plant tissue with enzyme preparations such as crude pectinase or polygalacturonase, which loosen the attachment between individual cells in a tissue. Zaitlin first used this technique in 1959 to separate viable cells from tobacco leaves. Methods of isolation have been described by Takebe *et al.* (1968); Servaites and Ogren (1977) and Dow and Callow (1979). Cells isolated in this way can be suspended in culture medium and remain metabolically active.

Separated cells from leaf tissue of tobacco pre-infected with Tobacco Mosaic Virus have been used to study the formation of viral RNA's in the infected cells, and for studies on the interaction between leaf tissue cells and elicitor chemicals produced by fungal pathogens (Dow and Callow, 1979). Button and Botha (1975) produced a suspension of single cells of *Citrus* by macerating callus with 2-3% Macerace enzyme: the degree of dispersion of cells from suspension cultures can also be improved by enzyme addition (Street, 1977c).

3.3.3. Protoplasts

A protoplast is the living part of a plant cell, consisting of the cytoplasm and nucleus with the cell wall removed. Protoplasts can be isolated from whole plant organs or tissue cultures. If they are then

placed in a suitable nutrient medium, they can be induced to re-form a cell wall and divide. A small cluster of cells eventually arises from each cell and, providing the protoplasts were originally plated at a relatively low density, can be recognised as one of many discrete 'callus colonies'. Plants can often be regenerated from such callus. Protoplast culture therefore provides one route whereby plants can be multiplied, but it is not yet used for routine micropropagation work, although the number of species in which plant regeneration has been achieved is steadily increasing.

At present isolated protoplasts are used chiefly in research into plant virus infections, and for modifying the genetic information of the cell by inserting selected DNA fragments. Protoplasts may also be fused together to create plant cell hybrids. Genetically modified cells will be only of general practical value if whole plants having the new genetic constitution can be regenerated from them. The ability to recover plants from protoplast cultures is therefore of vital importance to the success of such genetic engineering projects in plant science.

Methods of protoplast preparation. There are several different methods by which protoplasts may be isolated:

- by mechanically cutting or breaking open the cell wall;
- by digesting away the cell wall with enzymes;
- by a combination of mechanical and enzymatic separation.

For successful isolation it has been found essential to cause the protoplast to contract away from the cell wall, to which, when the cell is turgid, it is tightly adpressed. Contraction is achieved by plasmolysing cells with solutions of salts such as potassium chloride and magnesium sulphate, or with sugars or sugar alcohols (particularly mannitol) (see Chapter 4). These osmotica must be of sufficient concentration to cause shrinkage of the protoplasm, but of insufficient strength to cause cellular damage.

In the past, protoplasts have been mechanically isolated from pieces of sectioned plant material, but only very small numbers were obtained intact and undamaged. This method has therefore been almost completely replaced by enzymatic isolation techniques. Commercially available preparations used for protoplast isolation are often mixtures of enzymes from a fungal or bacterial source, and have pectinase, cellulase and/or hemicellulase activity: they derive part of their effectiveness from being of mixed composition (Evans and Cocking, 1977).

Protoplasts are usually isolated using a combination of several different commercial products. Plasmolysis helps to protect the protoplast when the cell wall is ruptured during mechanical separation and also appears to make the cell more resistant to the toxic effects of the enzymes used for cell wall digestion. It also severs the plasmodesmata linking adjacent cells and so prevents the amalgamation of protoplasts when the cell walls are digested away.

Tissue from an entire plant to be used for protoplast separation, is first surface sterilised. Some further preparation to allow the penetration of osmotic solutions and the cell wall degrading enzymes, is often advantageous. For instance, when protoplasts are to be separated from leaf mesophyll, the epidermis of the leaf is first peeled away, or the leaf is cut in strips and the tissue segments are then plasmolysed. The next step is to incubate the tissue with pectinase and cellulase enzymes for up to 18 hours in the same osmoticum, during which time the cell walls are degraded. Agitation of the incubated medium after this interval causes protoplasts to be released. They are washed and separated in solutions of suitable osmotic potential before being transferred to a culture medium.

Less severe and prolonged enzymatic cell digestion is required if plant tissue is first treated to mild mechanical homogenisation before cellulase treatment. Another technique calls for the sequential use of enzymes; firstly pectinase to separate the cells, and then, when separation is complete, cellulase to digest the cell walls. The yield of viable protoplasts can sometimes be increased by pre-treatment of the chosen tissue with growth substances before separation is attempted (Kirby and Cheng, 1979). Protoplasts are also commonly isolated by enzymatic treatment of organs or tissues that have been cultured *in vitro*. Cells from suspension cultures, which have been subcultured frequently, and are dividing rapidly, are one suitable source.

The successful isolation of viable protoplasts capable of cell division and growth, can depend on the manner in which the mother plant was grown. For example, Durand (1979) found that consistently successful protoplast isolation from haploid *Nicotiana glauca* plants depended on having reproducible batches of young plants *in vitro*. The composition of the medium on which these plants were cultured had a striking effect on protoplast yield and on their ability to divide. A low salt medium devoid of vitamins was particularly disadvantageous.

The light intensity under which the plants were grown was also critical.

Protoplast culture. Isolated plant protoplasts are very fragile and particularly liable to either physical or chemical damage. Thus if they are suspended in a liquid medium, it must not be agitated, and the high osmotic potential of the medium in which isolation was carried out must be temporarily maintained. As growth depends on adequate aeration, protoplasts are usually cultured in very shallow containers of liquid or solid media; fairly high plating densities (5×10^4 to 10^5 protoplasts/ml) may be necessary, possibly because endogenous chemicals are liable to leak away from such unprotected cells. To promote growth, it may also be beneficial to add to the medium supplementary chemicals and growth factors not normally required for the culture of intact cells.

The capability of plant protoplasts to divide appears to be closely related to their ability to form a cell wall (Meyer and Abel, 1975a,b). The type of wall that is produced initially can be controlled to some extent by the nature of the culture medium. A non-rigid wall can be produced on tobacco mesophyll protoplasts, for example, by culture in a medium containing a relatively high concentration of salts; but although such cells will divide 2–3 times, further cell division does not occur unless a rigid wall is induced to be formed by a change in the culture medium (Meyer, 1974). Under favourable circumstances formation of a cell wall seems to occur as soon as protoplasts are removed from hydrolysing enzyme preparations, and the first signs of cellulose deposition can be detected after only about 16 hours in culture medium. Once wall formation is initiated, the concentration of osmoticum is reduced to favour cell growth. This is readily accomplished in a liquid medium, but where protoplasts have been plated onto a solidified medium it will be necessary to transfer the cells on blocks of agar, to another substrate.

When it has formed a cell wall, the regenerated plant cell generally increases in size and may divide in 3–5 days. If further cell divisions occur, each protoplast gives rise to a small group of intact cells and then a small callus colony. Green chloroplasts in cells derived from leaf mesophyll protoplasts, lose their integrity and disappear as callus formation proceeds. Protoplasts may originate from cells of the intact plant, which are not all of the same genetic composition. If such cells are grown in liquid medium, they may stick together and form common cell walls. Colonies of mixed callus will result which

could give rise to genetically different plants (see Chapter 3) or plant chimeras (D'Amato, 1978).

To avoid cell aggregation, protoplasts should be freely dispersed and cultured at as low a density as possible. This may mean that, as in the culture of intact cells at low density (see above), nurse tissue, or a conditioned or specially supplemented medium, must be employed. A method of the latter kind was devised by Raveh *et al.* (1973). A fabric support has been used to suspend protoplasts in a liquid medium so that media changes can be made readily (Kirby and Cheng, 1979).

For further information, readers should consult one or other of the following references:

- Bajaj (1977), Evans and Cocking (1977) and Evans and Bravo (1983), who provide good basic reviews of the subject
- Gamborg *et al.* (1981), describe methods and protocols for protoplast isolation, culture (and fusion)
- Constabel (1982) and Fowke (1982a), chapters describing methods and equipment for protoplast isolation and culture

An entire plant was first regenerated from callus originated from an isolated protoplast in 1971 (Takebe *et al.*, 1971). Since then plants have been produced from the protoplasts of a wide range of species, using indirect shoot morphogenesis or indirect embryogenesis (Davey and Power, 1988). The direct formation of somatic embryos (see below) from cultured protoplasts is also possible (Zapata and Sink, 1980).

Protoplast fusion. Although fusion of plant protoplasts was observed many years ago, it has become especially significant since methods have been developed for protoplast isolation and subsequent regeneration into intact plants. Isolated protoplasts do not normally fuse together because they carry a superficial negative charge causing them to repel one another. Various techniques have been discovered to induce fusion to take place. Two of the most successful techniques are the addition of polyethylene glycol (PEG) in the presence of a high concentration of calcium ions and a pH between 8-10, and the application of short pulses of direct electrical current (electro-fusion). By mixing protoplasts from plants of two different species or genera, fusions may be accomplished:

- (a) between protoplasts of the same plant where fusion of the nuclei of two cells would give rise to a homokaryon (synkaryon);
- (b) between protoplasts of the same plant species (intravarietal or intraspecific fusion);
- (c) between protoplasts of different plant species or genera (interspecific or intergeneric fusion).

Fusions of types (b) and (c) above can result in the formation of genetic hybrids (heterokaryocytes), which formally could only be obtained rarely through sexual crossings. By separating the fused hybrid cells from the mixed protoplast population before culture, or by devising a method whereby the cells arising from fused cells may be recognised once they have commenced growth, it has been possible to regenerate new somatic hybrid (as opposed to sexually hybrid) plants. Some novel interspecific and intergeneric hybrid plants have been obtained by this means. A fusion of the cytoplasm of one kind of plant with the nucleus of another is also possible. Such cybrid plants can be useful in plant breeding programmes for the transfer of cytoplasmic genes.

The following references give further details about this research topic and its implications for crop improvement:

- Schieder and Vasil (1980). A well-referenced review which lists somatic hybrid cell lines or plants obtained by protoplast fusion.
- Ferenczy and Farkas (1980) is a book on protoplast research in fungi, yeasts and plants. Several papers describe the results of fusions between protoplasts of different plant species or genera.
- Dodds and Roberts (1982), a short chapter describing methods and techniques.
- Keller *et al.* (1982), a useful review of the production and characterisation of somatic hybrids and the practical applications of protoplast fusion technology.
- Kao (1982) and Fowke (1982a,b) describe protocols for protoplast fusions in great detail.
- Mantell *et al.* (1985). An introduction to plant genetic engineering of various kinds.
- Glimelius (1988). Uses of protoplast fusion for plant breeding objectives.
- Davey and Power (1988). Progress in protoplast culture, fusion and plant regeneration

4. CYTODIFFERENTIATION

In an intact plant there are many kinds of cells all having different forms and functions. Meristematic

cells, and soft thin-walled parenchymatous tissue, are said to be undifferentiated, while specialised cells are

said to be differentiated. The cells of callus and suspension cultures are mainly undifferentiated, and it is not yet possible to induce them to become of just one differentiated type. This is partly because culture systems are usually designed to promote cell growth: differentiation frequently occurs as cells cease to divide actively and become quiescent. Furthermore, the formation of differentiated cells appears to be correlated with organ development, therefore the prior expression of genes governing organogenesis may often be required. The *in vitro* environment can also be very different to that in the whole plant where each cell is governed by the restraint and influence of other surrounding cells. In suspension cultures, for example, cells are largely deprived of directional signals, influences from neighbouring differentiated tissues, and correlative messages that may normally pass between adjacent cells by way of interconnecting strands of protoplasm (plasmodesmata).

The differentiated state is also difficult to preserve when cells are isolated from a plant. Askani and Beiderbeck (1988) tried to keep mesophyll cells in a differentiated state. The character of palisade parenchyma cells with regard to size, cell form, colour and size, and distribution of chloroplasts could be preserved for 168h, but after this the chloroplasts became light green, their distribution was no longer homogeneous and some of the cells began to divide. Differentiated cells are most effectively produced *in vitro* within organs such as shoots and roots; even here there may not be the full range of cell types found in intact plants *in vivo*.

4.1. DIFFERENTIATED CELLS IN CALLUS AND CELL CULTURES

Three types of differentiated cells are commonly found in callus and cell cultures; these are vessels and tracheids (the cells from which the water-conducting vascular xylem is constructed), and cells containing chloroplasts (organelles carrying the green photosynthetic pigment, chlorophyll). Phloem sieve tubes may be present but are difficult to distinguish from undifferentiated cells.

4.1.1. Tracheid formation

Callus cultures are more likely to contain tracheids than any other kind of differentiated cell. The proportion formed depends on the species from which the culture originated and especially upon the kind of sugar and growth regulators added to the medium. This is discussed further in Chapter 10.

Tracheid formation may represent or be associated with an early stage in the development of shoot meristems. Nodules containing xylem elements in callus of *Pelargonium* have, for example, been observed to develop into shoots when moved to an auxin-free medium (Chen and Galston, 1967; Cassells, 1979).

The rapid cell division initiated when tissue is transferred to a nutrient medium usually occurs in meristems formed around the periphery of the explant. Cell differentiation does not take place in callus cultures during this phase but begins when peripheral meristematic activity is replaced or supplemented by the formation of centres of cell division deeper in the tissue. These internal centres generally take the form of meristematic nodules that may produce further expanded and undifferentiated cells (so contributing to callus growth) or cells that differentiate into xylem or phloem elements. Nodules can form primitive vascular bundles, with the xylem occurring centrally and the phloem peripherally, separated from the xylem by a meristematic region.

4.1.2. Chloroplast differentiation

The formation and maintenance of green chloroplasts in cultured plant cells represents another form of cellular differentiation which is easy to monitor, and which has been studied fairly extensively. When chloroplast-containing cells from an intact plant are transferred to a nutrient medium they begin to dedifferentiate. This process continues in the event of cell division and results in a loss of structure of the membranes containing chlorophyll (thylakoids) and the stacks (grana) into which they are arranged, and the accumulation of lipid-containing globules. The chloroplasts eventually change shape and degenerate.

Callus cells frequently do not contain chloroplasts but only plastids containing starch grains in which a slightly-developed lamellar system may be apparent. All the same, many calluses have been discovered that do turn green on continued exposure to light and are composed of a majority of chloroplast-containing cells. Chloroplast formation can also be connected with the capacity of callus to undergo morphogenesis. Green spots sometimes appear on some calluses and it is from these areas that new shoots arise. By subculturing areas with green spots, a highly morphogenic tissue can sometimes be obtained. The formation of chloroplasts and their continued integrity is also favoured by cell aggregation. When

green callus tissue is used to initiate suspension cultures, the number of chloroplasts and their degree of differentiation are reduced. Nevertheless, there can be some increase in chlorophyll content during the stationary phase of batch cultures.

The level of chlorophyll so far obtained in tissue cultures is well below that found in mesophyll cells of whole plants of the same species, and the rate of chlorophyll formation on exposure of cultured cells to the light is extremely slow compared to the response of etiolated organised tissues. The greening of cultures also tends to be unpredictable and even within individual cells, a range in the degree of chloroplast development is often found. In the carbon dioxide concentrations found in culture vessels, green callus tissue is normally photomixotrophic (i.e. the chloroplasts are able to fix part of the carbon that the cells require) and growth is still partly dependent on the incorporation of sucrose

into the medium (Vasil and Hildebrandt, 1966). However, green photoautotrophic callus cultures have been obtained from several different kinds of plants. When grown at high carbon dioxide concentrations (1–5%), without a carbon source in the medium, they are capable of increasing in dry weight by photosynthetic carbon assimilation alone (see Street, 1977a).

Photoautotrophic cell suspensions have also been obtained. They too normally require high carbon dioxide levels, but cell lines of some species have been isolated capable of growing in ambient CO₂ concentration (Xu *et al.*, 1988). Why cultured cells do not freely develop fully functional chloroplasts is not fully known. Some hypotheses have been summarised by Dalton (1980). The cytology of chloroplast formation is described in Yeoman and Street (1977). Photoautotrophic growth of shoots is described in Chapter 2.

5. MORPHOGENESIS

5.1. NATURE AND INDUCTION

New organs such as shoots and roots can be induced to form on cultured plant tissues. Such freshly originated organs are said to be adventive or adventitious. The creation of new form and organisation, where previously it was lacking, is termed morphogenesis or organogenesis. Tissues or organs that have the capacity for morphogenesis/organogenesis are said to be morphogenic (morphogenetic) or organogenic (organogenetic). So far it has been possible to obtain the *de novo* (adventitious) formation of:

- shoots (caulogenesis) and roots (rhizogenesis) separately. The formation of leaves adventitiously *in vitro* usually denotes the presence of a shoot meristem. Sometimes leaves appear without apparent shoot formation: opinions are divided on whether such leaves can have arisen *de novo*, or whether a shoot meristem must have been present first of all and subsequently failed to develop.

- embryos that are structurally similar to the embryos found in true seeds. Such embryos often develop a region equivalent to the suspensor of zygotic embryos and, unlike shoot or root buds, come to have both a shoot and a root pole. To distinguish them from zygotic or seed embryos, embryos produced from cells or tissues of the plant body are called *somatic embryos* (or *embryoids*) and the process leading to their inception is termed *embryogenesis*. The word '*embryoid*' has been especially used when it has been unclear whether the embryo-like structures seen in cultures were truly the somatic equivalent of zygotic embryos. Somatic embryogenesis is now such a widely observed and documented event that *somatic embryo* has become the preferred term.

- flowers, flower initials or perianth parts. The formation of flowers or floral parts is rare, occurring only under special circumstances and is not relevant to plant propagation.

6. HAPLOID PLANTS

6.1. ANTHR AND POLLEN CULTURE

In 1953 Tulecke discovered that haploid tissue (i.e. tissue composed of cells having half the chromosome number that is characteristic of a species), could be produced by the culture of *Ginkgo* pollen. Little notice was taken of his work until Guha

and Maheshwari (1964, 1967) managed to regenerate haploid plants from pollen of *Datura innoxia* by culturing intact anthers. Since then a great deal of research has been devoted to the subject.

The basis of pollen and anther culture is that on an appropriate medium the pollen microspores of some