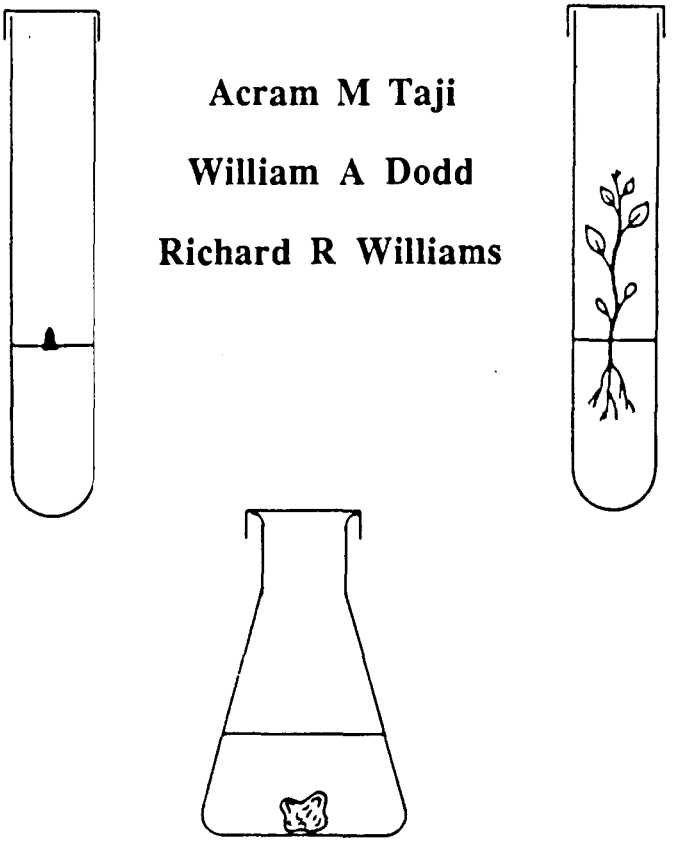
PLANT TISSUE CULTURE PRACTICE



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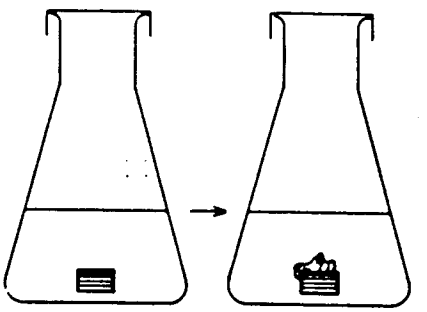

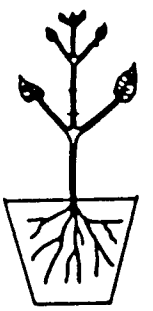

PREFACE

The propagation of higher plants in vitro, one of the most important aims of plant tissue culture, has shown a dramatic development in Australia and elsewhere since 1976 when Dr Ron de Fossard, the father of Plant Tissue Culture in Australia, produced his book "Tissue Culture for Plant Propagators". His book remains a classic in the field of plant tissue culture. His systematic method of media preparation and testing revolutionized our approach to tissue culture.

The purpose of this book is to provide a practical reference to the production of higher plants via in vitro techniques. The book is aimed at researchers, students, nurserymen and other individuals who are interested in plant cell and tissue culture, particularly in clonal propagation.

Basic botanical knowledge is critical to success with in vitro techniques, therefore the main body of the text includes a chapter covering basic plant anatomy and morphology . Theoretical and practical aspects of plant tissue culture are covered in Chapters 2-5 with specific attention to micropropagation.

Appendices provide an easy reference to technical information and solutions to many of the problems one may encounter in the course of tissue culture. Many recipes, arising from our own work with Australian Native Plants, are also included here.

We hope that this book will provide a valuable guide to the field of tissue culture from a practical perspective.

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SOME ABBREVIATIONS USED IN PLANT TISSUE CULTURE

ABA	abscisic acid
ADP	adenosine diphosphate
ATP	adenosine triphosphate
B5	the medium of Gamborg *et al.* (1968)
BA	benzyl adenine see BAP
BAP	benzylamino purine
BPA(PBA)	6-Benzylamino-9-(2-Tetrahydropyranyl) 9H purine
CW	coconut water
2,4-D	2,4-dichlorophenoxyacetic acid, a synthetic auxin
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetic acid
FW	fresh weight
HEPA	refers to the "high efficiency particulate air" filter
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
2iP	N^6-isopentenyladenine = $\gamma.\gamma$ - (dimethylallyl) aminopurine, the naturally occurring cytokinin
K	see KIN
KPa	kilopascals
KIN	N^6-furfuryladenine (Kinetin) = N^6 - furfurylaminopurine, a synthetic cytokinin
M	Molar
mg	milligram
$mg.l^{-1}$	milligrams per litre, (mg/L)
mM	millimolar
MS	Murashige and Skoog (1962) medium
µmole	micromole
NAA	naphthalene acetic acid
NOA	naphthoxy acetic acid
nm	nanometer
PCV	packed cell volume
PEG	polyethylene glycol
pH	indicates degree of acidity or alkalinity on a scale of 1 to 14
PPFD	Photosynthetic Photon Flux Density
ppm	parts per million
RNA	ribonucleic acid
2,4,5-T	2,4,5-trichlorophenoxyacetic acid, a synthetic auxin
UV	ultraviolet light
v/v	volume to volume
w/v	weight in volume
WPM	woody plant medium of McCowan & Lloyd (1980)
Zeatin	6-(4-hydroxy-3-methyl-2-butenylamino) purine

CHAPTER I

1. Introduction

Plant tissue culture is the growing of microbe-free plant material in an aseptic environment such as sterilized nutrient medium in a test tube and includes Plant Protoplast, Plant Cell, Plant Tissue and Plant Organ Culture. Plant tissue culture techniques have, in recent years, developed into a very powerful tool for propagation of many plant species. The technology had its beginning with the German Scientist Haberlandt's speculation regarding cell totipotency at the turn of the 20th century. Haberlandt suggested that techniques for isolating and culturing plant tissues should be developed and postulated that if the environment and nutrition of cultured cells were manipulated, those cells would develop into a normal plant.

Plant tissues were first successfully cultured by White in 1934. By 1939, White had reported the first successful callus culture of carrots and tobacco. In 1957, a key paper by Skoog and Miller was published in which they proposed that quantitative interactions between auxins and cytokinins determine the type of growth and morphogenic event that would occur. Their studies with tobacco indicated that high auxin to cytokinin ratios induced rooting while the reverse induced shoot morphogenesis. Unfortunately this pattern of response is not universal. While manipulations of auxins to cytokinins ratio have been successful in obtaining morphogenesis in many taxa, it is now clear that many other factors affect the ability of cells in culture to differentiate into roots, shoots, or embryos.

A major stimulus for application of plant tissue culture techniques to the propagation of many species may be attributed to the early work by Morel on the propagation of orchids in culture in 1960, and to the development and widespread use of a new medium with a high concentration of mineral salts, by Murashige and Skoog in 1962.

Basic botanical knowledge is central to success in plant tissue culture techniques. In this chapter we endeavour to provide you with some understanding of anatomy of flowering plants.

2. Anatomy

2.1 Seed and seedling development

The mature embryo of flowering plants has either one cotyledon (monocotyledonous plants) or two cotyledons (dicotyledonous plants). The cotyledons, sometimes referred to as the seed leaves, are the first leaves of the young plant.

A seed may be defined as a mature (ripened) ovule. Each typical seed consists essentially of protective seed coat(s), some form of stored food plus an embryo. When a seed germinates the embryo enlarges (grows), the seed coat(s) bursts, and the young plant emerges. Early growth is dependent upon food stored within the endosperm or, if there is no endosperm, within the cotyledons. During germination the primary root arises from the radicle (root primordium) and is the first embryo structure to emerge. Lateral branches may soon appear on the primary root. The young stem, arising from the stem tip of the plumule, follows the root in emerging from the seed. Young leaves are soon formed, and the seedling plant develops rapidly from this point (Figs. 1 and 2).

The region of a seedling below the cotyledons, which gradually merges into the radicle or root, is the hypocotyl; the region immediately above the cotyledons is the epicotyl.

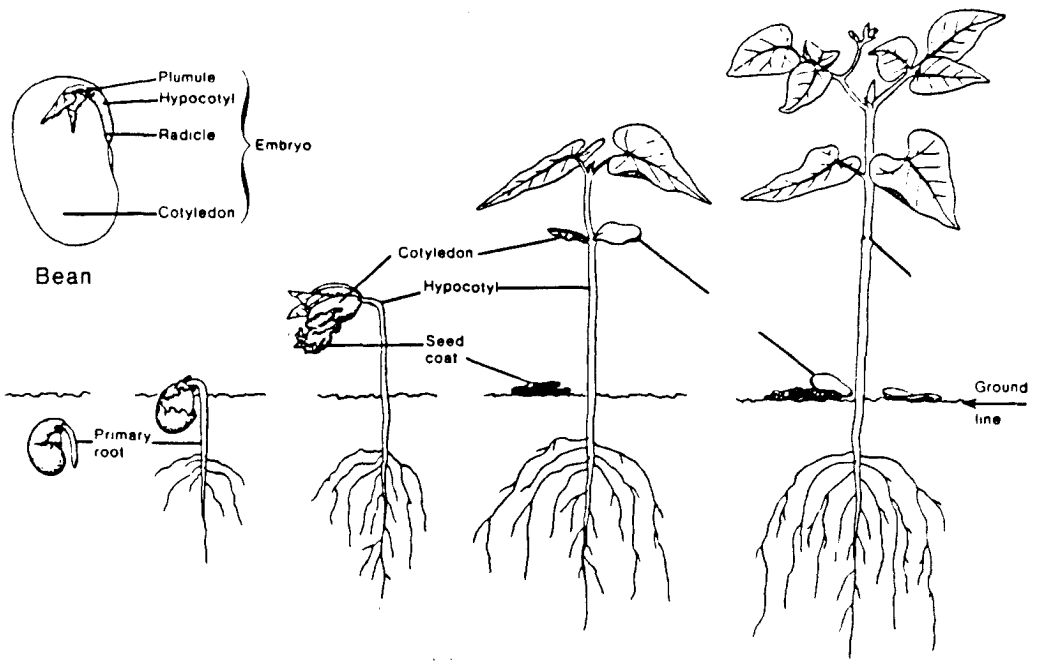


Fig. 1 Seedling development in bean (*Phaseolus vulgaris*) a dicotyledonous plant.

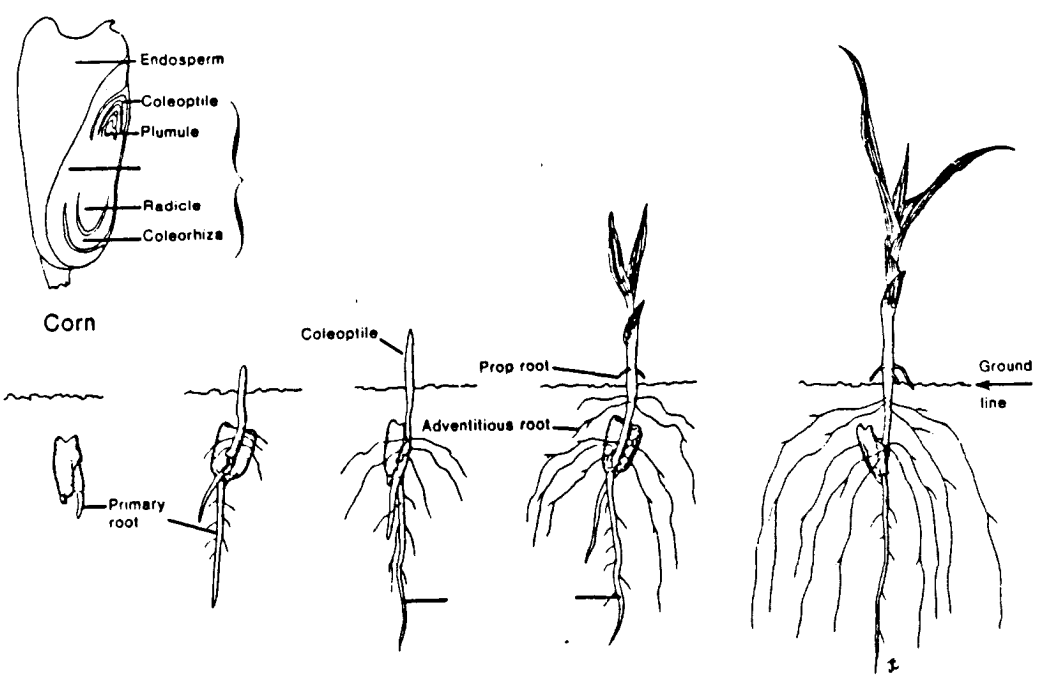
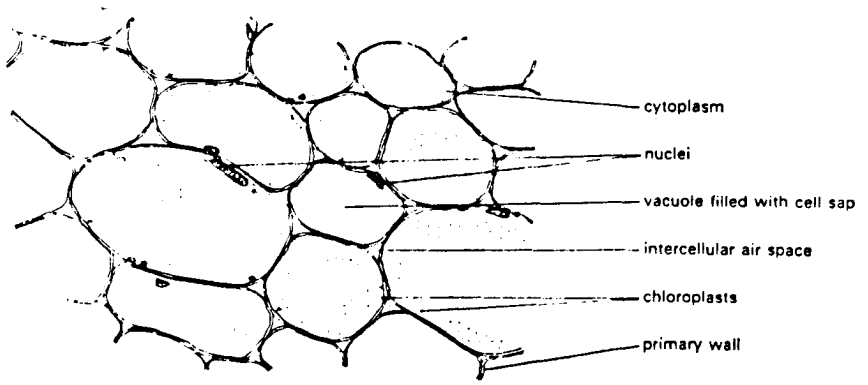


Fig. 2 Seedling development in corn (*Zea mays*) a monoctyledonous plant.

2.2 Basic Tissues

(a) Parenchyma

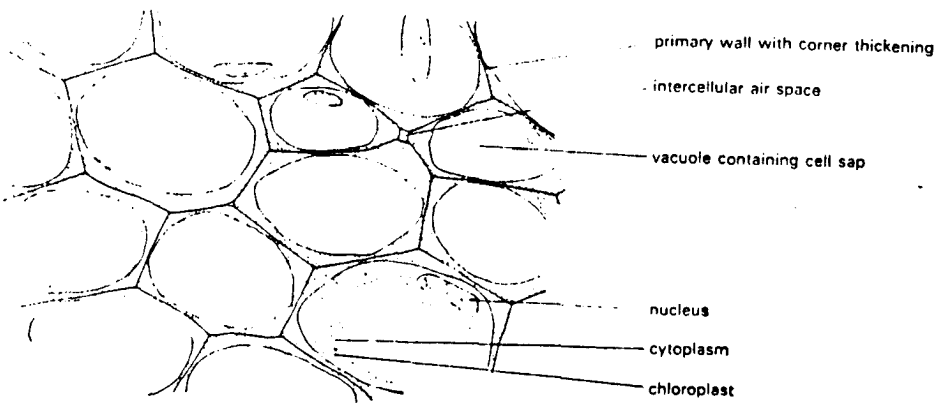
Parenchyma is the most common and perhaps least specialised or differentiated of plant tissues. Much of the non-structural carbohydrate and water stored by the plant is found in parenchyma. A typical parenchyma cell has dimensions which are as wide as long (**isodiametric**) and an active **protoplast** enclosed by a thin cellulose **primary cell wall. Intercellular spaces** between cells are common in parenchyma.



(b) Collenchyma

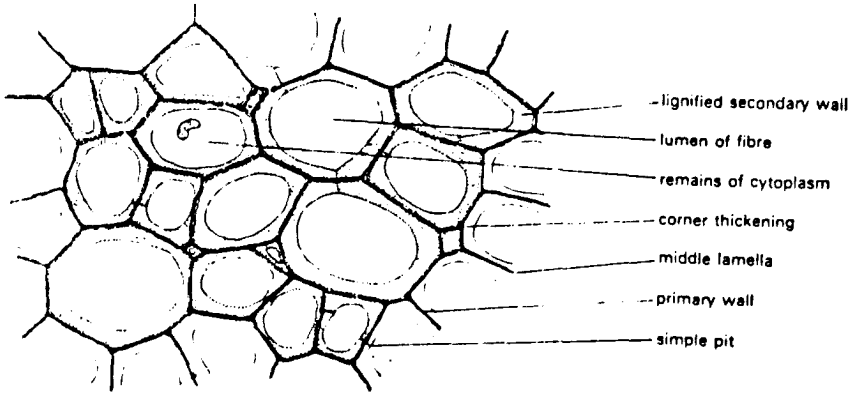
Collenchyma consists of cells similar to parenchyma but with extensive thickening of the primary cell wall. It is generally located in the peripheral portion of the stem and in various parts of the leaf. The flexible and plastic cell walls of collenchyma provide adequate support for its neighbouring cells. Since collenchyma seldom produces secondary walls it represents cells with extensively thickened primary walls.

The close relationship between collenchyma and parenchyma tissues is apparent in stems where both tissues lie adjacent to each other. In many instances there is no definite line of demarcation between them, because cells having walls with intermediate thickness occur between the two distinct kinds of tissue.



(c) Sclerenchyma

Sclerenchyma is the main support tissue in plants. Lignin thickening is laid down on the secondary and primary cell walls and the walls become so thick that there is little room for the protoplast which disappears at maturity. The cells comprising sclerenchyma tissue may be of two types, **fibres** or **sclereids**. The fibres are typically greatly elongated with sharply pointed end walls in Longitudinal Sections (L.S.) but the sclereids, by contrast, are small and dumpy and are variable in shape. They occur in hard layers of fruits and seeds. The grittyness in the flesh of a pear is due to the stone cells (sclereids).



2.3 Vascular Tissues

The **vascular tissues** conduct water and solutes around the plant; **xylem** is concerned with water transport and **phloem** with transport of soluble organic materials. Both xylem and phloem consist of several cell types. In primary stems these tissues are located in vascular bundles often with the phloem to the outside and the xylem to the inside. The phloem and xylem are separated by a few rows of thin walled meristematic cells called **cambium.**

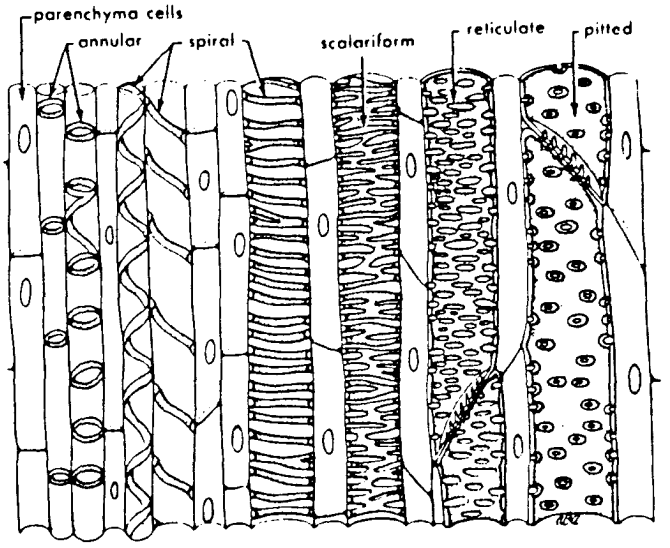
(a) Xylem

There are four types of cells found in xylem in different proportions: **vessels, tracheids, fibres** and **parenchyma.** The most characteristic xylem cells are vessels and tracheids which have thick **lignified** walls and are the water conducting elements. The tracheids are long, elongated cells similar to fibres except that they have a larger diameter. In Transverse Sections (T.S.) the vessels are the large, almost circular cells in the xylem tissue. Tracheids are hard to distinguish from fibres or vessels (except for the size difference) in T.S. Within xylem the following distinctions can be made in T.S.

Metaxylem vessels - the largest cells found in the last formed portion of the xylem.

Protoxylem vessels - the first formed lignified cells, usually somewhat crushed or displaced (by stretching during elongation). Note that in the stem, the **protoxylem** is to the **inside** of the **metaxylem.** i.e. **endarch** position whereas in the root, the protoxylem is on the outside (**exarch**).

The lignified thickening of the tracheids and vessels can be one of five types. **Annular** or **spiral thickening** is characteristic of protoxylem vessels whereas **scalariform, reticulate,** or **pitted thickening** is characteristic of metaxylem.

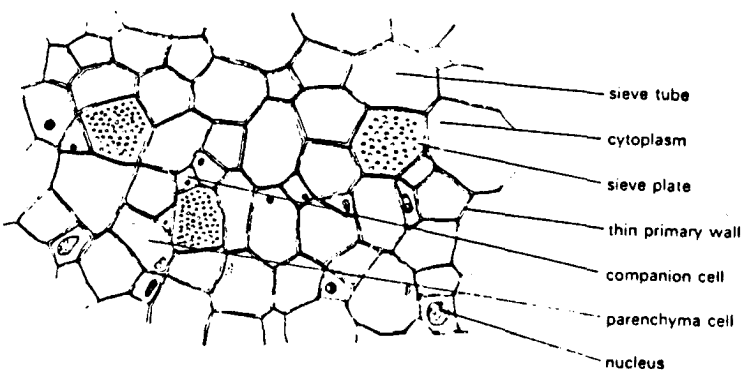


Protoxylem **Metaxylem**

Parenchyma cells - small thin-walled cells associated with the vessels and tracheids.

(b) Phloem:

Four types of cell may be found in the phloem: **sieve tube members, companion cells, parenchyma cells** and **fibres.** Many cells appear tubular, elongated and thin walled. These are the sieve tube members. These cells are active when immature but as a **sieve tube member** matures the nucleus disintegrates though the cytoplasm remains. Each sieve tube member is accompanied by a **companion cell** which has a nucleus at maturity. The part of a wall of sieve tube member which bears one or more sieve areas is commonly called a **sieve plate.** Some of these may appear blocked. These blockages are called **slime plugs** and are accumulations of protein material in the pores. These plugs will prevent constant exudation from the sieve tubes.



3. Anatomy of main plant organs

Seed plants dominate the modern landscape. They include not only the cone-bearing trees (gymnosperms) such as the cycads, pines, cedars and spruces but also the everpresent, fruit bearing, flowering plants (angiosperms), which are the best known, the most common and the most numerous of seed plants. They are widely distributed throughout the world and are the plants humans depend upon for food, many building and industrial materials, fibres, fuel and drugs. Although flowering plants were first classified according to their growth form as trees (woody stems) or as herbs (non-woody stems) or according to their use as source of food, medicine, fibre or decoration, by 1700's they were classified by the characteristics of their flowers, fruits and seeds.

The flowering plants are divided into the **Dicotyledons** (Dicots) and **Monocotyledons** (Monocots). Each seed contains an embryo plant that has at least one specialized leaf, or cotyledon, modified for food storage or absorption. The number of seed leaves is a reliable characteristic in distinguishing one plant from another, but since it is within the seed and not readily visible, other characteristics are used as well.

1. The Dicots are characterised by having two cotyledons (seed leaves) in the embryo, flower parts mostly in fours and fives, a cambium with the vascular bundles forming a circle around the central pith, both woody and herbaceous forms, and leaves mostly with reticulated (net) veins. The Dicots are a much larger group than Monocots, and common examples include most trees and shrubs, e.g. eucalypts, carnations, potatoes.

2. The Monocots are characterised by having one cotyledon in the embryo, flower parts mostly in threes, no cambium, and the vascular bundles scattered throughout the stem (in the pith or ground tissue). Nearly all are herbaceous forms with leaves, mostly parallel veined. Common examples include corn, bamboo, sugar cane, iris, lillies, orchids and palms.

A typical seed plant (either dicot or monocot) is composed of a number of interrelated parts, each having different structures and functions but each part contributing to the unity of the plant as a whole. The major parts of a seed plant are leaves, stems, roots, flowers, fruits and seeds. The leaf, stem and root are the vegetative parts concerned with the growth, nutrition, and development of a plant and are called **organs** - each composed of one or more **tissue** such as epidermis, cortex, xylem and phloem.

Flowers, fruits and seeds are composed of one or more different organs such as petals, pistils, anthers and ovules (Fig. 3).

3.1 The root

In most vascular plants, the roots constitute the underground portion of the sporophyte and are involved primarily in anchorage and absorption of water and minerals. Two other functions associated with roots are storage and conduction. Most roots are important storage organs and some, such as those of the carrot, sugarbeet and sweet potato are specifically adapted for the storage of food.

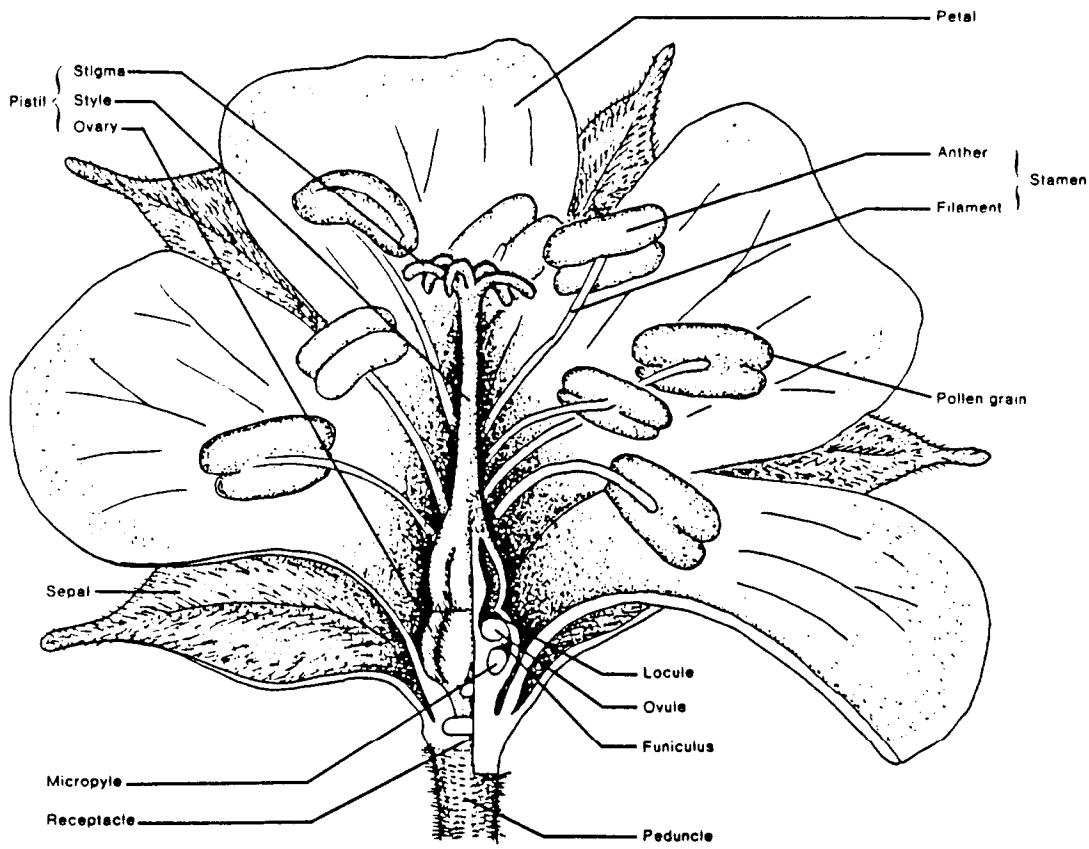


Fig. 3 Longitudinal Section of a dicotyledonous flower.

3.1.1 Organization of root apex

The apical meristem of a root is very similar to the shoot apical meristem in that it forms three meristematic areas, the **protoderm** (developing into the epidermis), the **procambium** (which develop into the stele) and the **ground meristem** (which forms the cortex); in addition, the root apical meristem forms cells ahead of its position which make up the root cap and serve for the protection of the root apical meristem as it pushes through the soil. The root system lacks a cuticle.

The cells of the protoderm elongate and vacuolate and, a short distance from the root tip, many of them produce an outgrowth which develops into a ROOT HAIR. These root hairs expand rapidly and twine around soil particles; their very thin walls absorb water (and mineral ions) freely. The **root hair zone** which is also called the PILIFEROUS LAYER of the root, increases the absorbing surface of the root enormously - one estimate is that a rapidly growing rye plant develops more than 5 kilometres of new roots and 100 kilometres of root hairs per day. The life span of root hairs is very short. In older roots, absorption ceases and the surface becomes cutinized.

Lateral roots originate from a group of cells (the PERICYCLE) deep within the root and opposite PROTOXYLEM tips; a small conical cell mass is initiated which grows at right angles to the main root-axis and, after a time, bursts through the epidermis. Its anatomy and organization is exactly the same as that of the main root.

3.1.2 Root anatomy (Fig. 4)

A cross-section of a root, examined microscopically, shows the following main features:

(1) THE EPIDERMIS OR PILIFEROUS LAYER WITH ROOT HAIRS: Root hairs are produced in the young region of the root behind the root apex, and may have shrivelled and disappeared if one is viewing an older part of the root.

(2) THE CORTEX: A broad, homogeneous region composed of thin-walled parenchyma cells, loosely packed together, with large intercellular air spaces; the cortical cells are often filled with starch grains, particularly in older parts of the root.

(3) THE ENDODERMIS: This is the innermost layer of the cortex and is usually a well-defined layer, one cell wide, its cells being distinguished by a band-like thickening in the radial walls, called the CASPARIAN STRIP.

(4) THE STELE: Consists of:

  (i) THE PERICYCLE: a layer of thin-walled cells immediately inside the endodermis; lateral roots arise in the pericycle at points opposite the protoxylem.

  (ii) XYLEM: consists of lignified, thick-walled elements in 3,4,5 or 7 radially-arranged groups in dicot roots, and in up to 30 groups in monocot roots. The smaller, first-formed xylem (PROTOXYLEM) elements lie towards the outside part of the stele whereas the later-formed METAXYLEM elements lie towards the centre of the root (termed exarch condition), though not always to the centre which might be occupied by a small parenchymatous pith. In contrast, in the stem, the PROTOXYLEM is to the inside of the METAXYLEM (endarch).

  (iii) PHLOEM: consists of as many groups as there are xylem points, lying between the protoxylem groups. The xylem and phloem thus lie on alternating radii in contrast to the situation in the stem where they lie on the same radius.

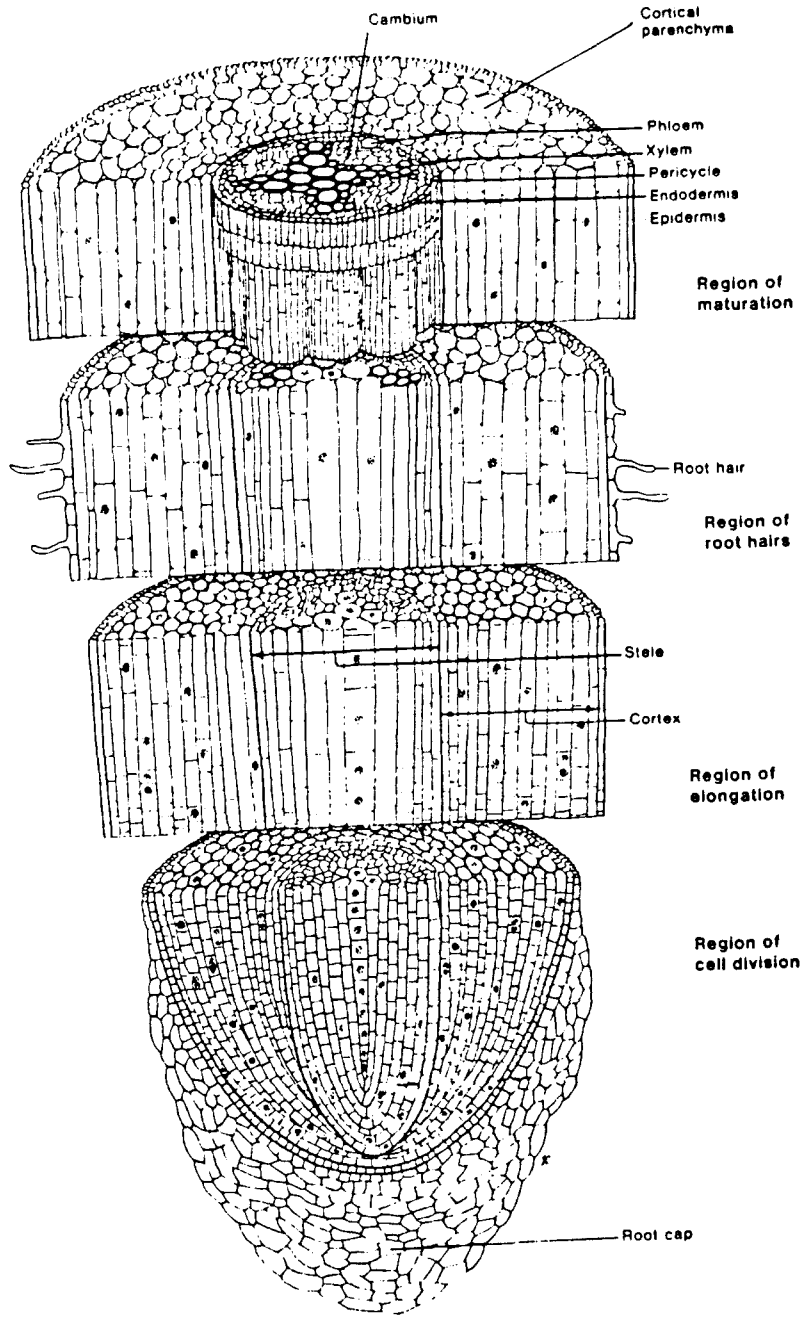


Fig. 4 A typical dicotyledonous root.

3.2 The stem (Fig. 5)

The stem of a typical plant functions primarily to support the leaves so that they are always exposed to sunlight. Flowers and fruits are also borne on the stem and its branches. The stem serves to conduct water and dissolved mineral salts upwards and to transport products of photosynthesis in leaves downwards. Many stems are greatly modified for food storage, others are the main photosynthetic organ of the plant, some are important in the vegetative propagation of the plant (asexual reproduction).

3.2.1 Organization of stem apex (Fig. 6)

The above ground parts of the plant arise from a mound of continuously dividing cells, the **apical meristem**, found in the tip of every stem and branch. The **primary tissues** of leaves, young stems and branches, vascular bundles and cortex all differentiate directly from cells derived from the stem tip. Secondary tissues usually originate from cells derived from the vascular or cork cambium. The perennial habit of woody stems is possible because of the meristematic nature of the cells in the stem apex.

Two regions are visible in the apical meristem.

(1) An outer 1-4 layered region where cell divisions are anti-clinal, i.e. perpendicular to the surface; this results essentially in an increase of surface area and little increase in depth; this region is called the **TUNICA**.

(2) Below the tunica, the cells divide in all directions giving an increase in tissue volume; this region is called the **CORPUS**.

If cells of both tunica and corpus divide at the same rate, this will result in larger surface layers and superficial folds will develop. The first adjustment to this is a periclinal division (i.e. parallel to the surface) in the second layer of the tunica, then more divisions and the formation of a distinct lump of tissue. This is the LEAF PRIMORDIUM or INITIAL; it divides rapidly and forms a small green organ. As the apex of the stem grows, new folds develop elsewhere on the apex, and new leaf primordia form. This process is responsible for the PHYLLOTAXIS of the shoot, the particular arrangement of leaves on the stem which is characteristic of individuals in a species. Leaves thus arise exogenously (i.e. from superficial tissues of the stem) in contrast to the endogenous (i.e. deep-seated) origin of lateral roots (from Pericycle). Axillary buds are also exogenous and arise in a very similar way to leaves; these buds have their own apical meristems, leaf and bud primordia, and later become dormant ready to grow if stimulated, for example, by removal of the main shoot tip (e.g. as in pruning). Apical meristems give rise to three meristematic areas, (1) a PROTODERM, which later gives rise to the EPIDERMIS, the outermost layer of cells of the shoot, (2) a PROCAMBIUM, which gives rise to VASCULAR BUNDLES, and (3) a GROUND MERISTEM, which gives rise to the CORTEX and PITH. The extreme smallness of the apical meristem makes it difficult to excise and to culture; usually, so-called "MERISTEM CULTURE" is in reality "SHOOT TIP CULTURE" where the explant consists of the apical meristem with at least one or two leaf primordia with axillary buds.

3.2.2 Stem anatomy

If a cross-section is cut through a stem a few centimetres from the shoot-apex and examined with a microscope, the following tissues are seen:

(1) THE EPIDERMIS: the single, outermost layer of parenchyma cells with cutin-impregnated outer walls, sometimes bearing multi-cellular or uni-cellular hairs at intervals.

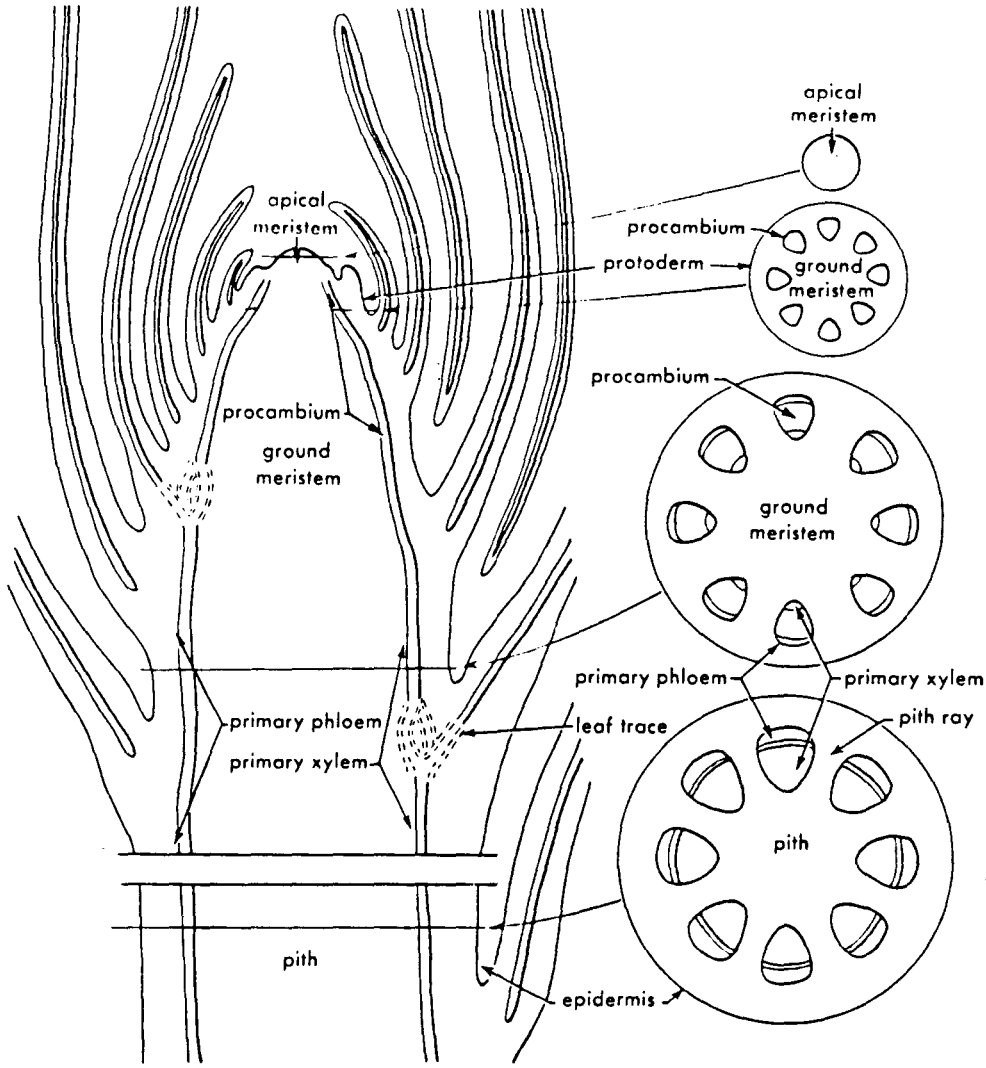


Fig. 5 Development of tissues of the primary plant body of a dicotyledonous plant.

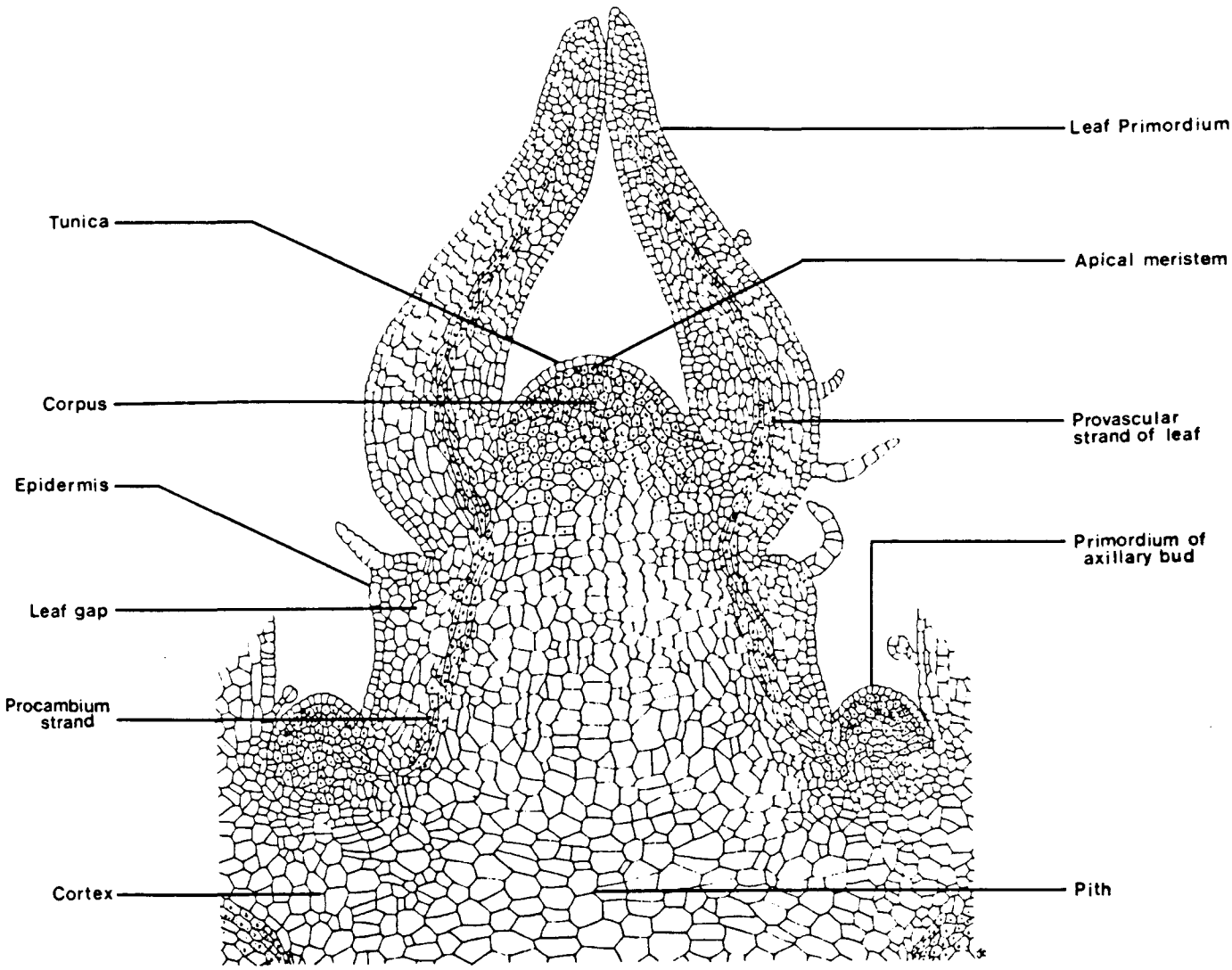


Fig. 6 Longitudinal Section of a shoot tip.

(2) THE CORTEX: which consists of large, thin-walled cells (parenchyma) with numerous intercellular air-spaces, and which might have an outer strengthening band of collenchyma.

(3) THE STELE: is the central cylinder occupying the remainder of the stem. It consists of:-

(i) A ring of vascular bundles, i.e. the strands of water- and food-conducting tissues;

(ii) The PITH (or MEDULLA) occupying the centre of the stem and composed of large, thin-walled parenchyma cells;

(iii) Rays - radiating out from the pith are small strands of parenchyma tissue, the MEDULLARY RAYS, which pass between the vascular bundles and link up with the cortex.

Each vascular bundle contains xylem and phloem and, in the case of dicotyledonous stems, a CAMBIAL ZONE, a meristematic region of 2-4 layers of small, thin-walled cells found between the xylem and the phloem; the CAMBIUM (a single layer of cells in the cambial zone) forms new cells some of which mature into xylem and some into phloem (Fig. 7).

Monocotyledonous stems differ from those of dicotyledons in that the vascular bundles typically are scattered throughout the stem and a cambium is usually lacking. This is the reason why monocotyledons cannot be propagated by budding and grafting. The secondary thickening characteristic of certain dicot stems usually does not occur in monocot stems and never in any case results in the large woody cylinder so typical of certain dicots (Fig. 8).

Monocots also have many more, but smaller, vascular bundles than do dicots, a reflection of their parallel venation.

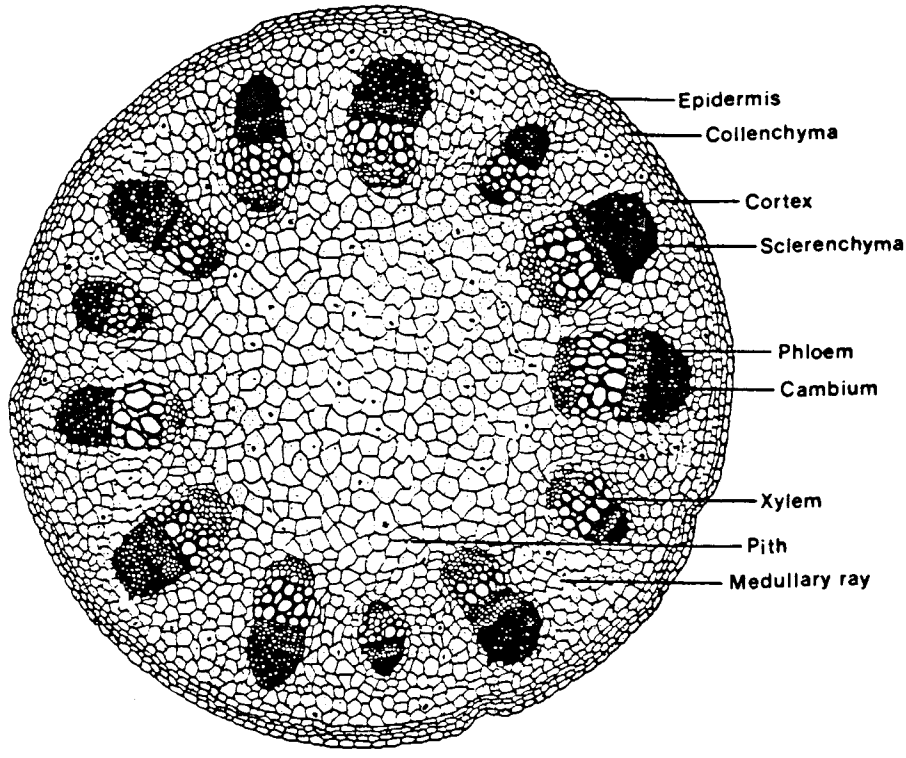


Fig. 7 Transverse section of a dicotyledonous stem.

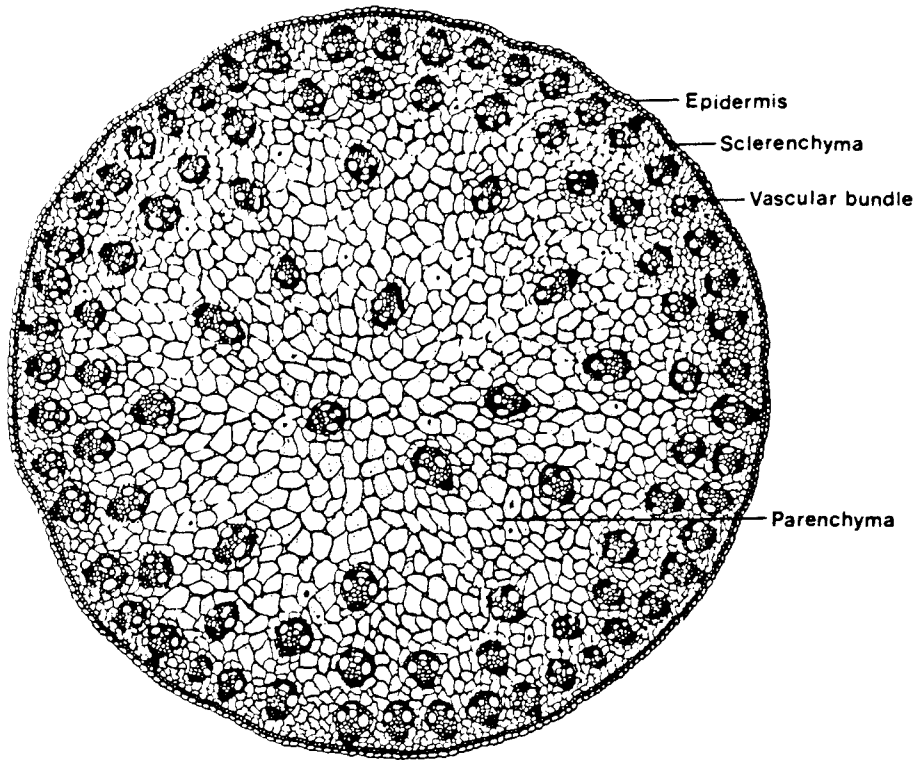


Fig. 8 Transverse section of a monoctyledonous stem.

3.3 Leaf anatomy

3.3.1 Dorsiventral Leaf (Fig. 9)

The leaves of many dicots (and some monocots) are **dorsiventral**, i.e. they have an upper (or **adaxial**) surface and a lower (or **abaxial**) surface, which are morphologically differentiated.

i. **Upper epidermis** composed of a single layer of cells, square-rectangular in outline, their outer walls covered by a cuticle, and containing **no chloroplasts.** Few stomata, if any, are present in upper epidermis.

ii. **Palisade mesophyll.** This lies directly beneath the upper epidermis and consists of one or more layers of rather narrow, closely packed thin-walled, rectangular cells with long axes perpendicular to the epidermis. Each cell contains a large number of chloroplasts. There is a well developed system of intercellular spaces throughout the tissue.

iii. **Spongy mesophyll.** This consists of thin-walled, loosely packed, irregularly-shaped cells between which are large **intercellular spaces.** These intercellular spaces communicate with the small ones among the palisade cells. Chloroplasts are present in the cells, but in smaller numbers than in the palisade cells.

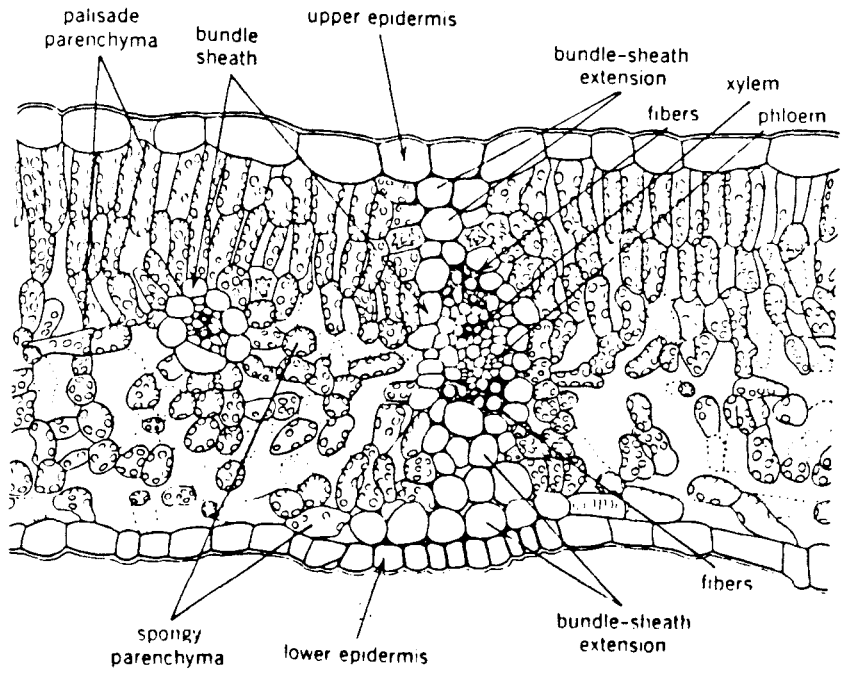


Fig. 9 Transverse Section of a dicotyledonous leaf - Dorsiventral.

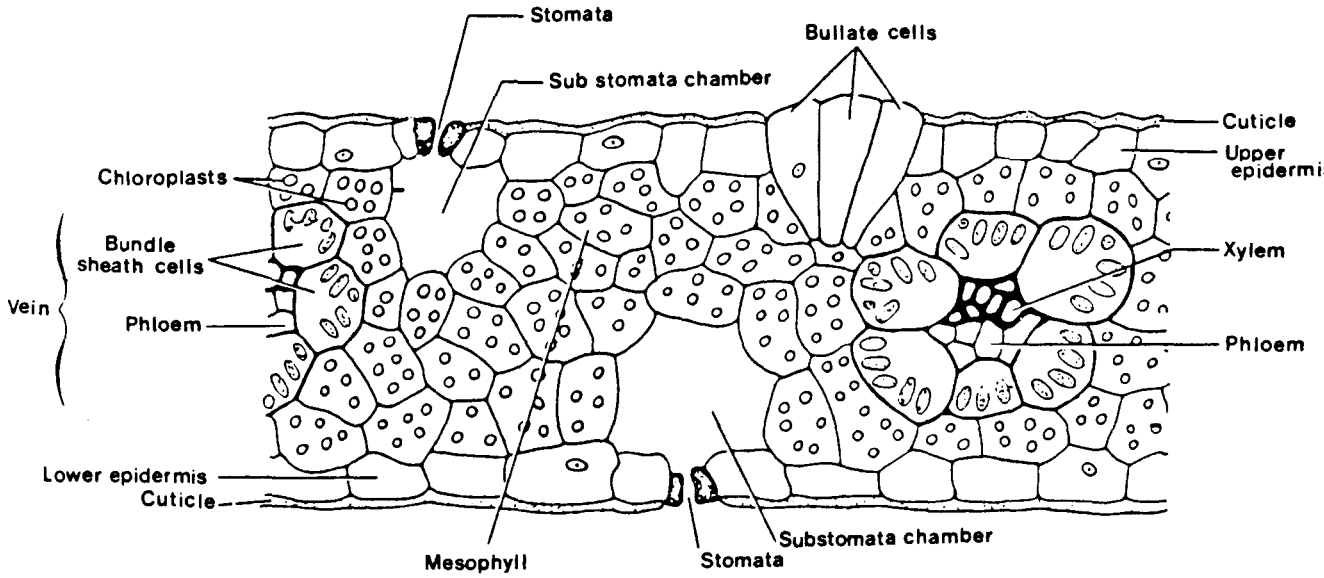


Fig. 10 Transverse Section of a monocotyledonous leaf - Isobilateral.

iv. **Lower epidermis**, similar in structure to the upper but differing from it in the numerous **stomata** present. Each stomatal pore opens into a large intercellular space.

v. **Vascular system.** A section through the **midrib** region can reveal the large crescent shaped mass of **xylem** towards the upper surface on the leaf and a **phloem** patch towards the **lower** surface. Above and below the vascular strand, next to the upper and lower epidermis, the mesophyll tissue is replaced by **collenchyma cells** which increase the mechanical rigidity of the leaf.

3.3.2 Isobilateral Leaf (Fig. 10)

An isobilateral leaf is morphologically the same on both sides though there is still an abaxial and an adaxial surface, which can be distinguished from each other in T.S. (Transverse Section) by the position of the xylem and the phloem of the vascular bundles. Leaves of this type are usually oriented so that there is approximately equal incidence of light rays on both adaxial and abaxial surfaces. The leaves of many monocots are isobilateral.

Appendix 17 provides useful information on microtechniques used in tissue culture.

CHAPTER 2

BENEFITS OF PLANT TISSUE CULTURE

The most common benefit ascribed to plant tissue culture is that of cloning or mass production of genetically identical organisms.

1. **Rapid multiplication of clones** - with true tissue or cell culture there may be thousands of cells in a single culture each capable of producing a whole plant, these plants will be genetically identical. Even with micropropagation or organ culture, many new shoots may be induced to grow from a single explant and each can be sub-cultured to produce a further multiplication soon giving large numbers of new individuals. A modest multiplication rate of 5 will give nearly 2 million plantlets in 9 generations which would typically take 9-12 months.

2. **Genetic uniformity** - Since tissue culture procedures are vegetative, the random re-combination of genetic characters associated with sexual (seed) propagation is avoided. Therefore, the resulting offspring are essentially genetically identical. This is important where uniformity of the crop is required or where it is desirable to ensure that a particular inheritable characteristic is maintained. Traditionally, cutting propagation or grafting is used because seedlings of many species are genetically quite variable. However, genetic consistency should not be assumed with tissue culture because mutations may occur as the cells multiply, particularly under the artificial conditions of the culture environment with high levels of hormones and nutrients. This genetic mutation during vegetative multiplication is called **"somatic variation"**.

3. **Aseptic conditions** - The process of in vitro culture demands aseptic conditions. In turn the maintenance of plant cultures under aseptic conditions provides a source of largely pathogen-free material which not only supplies healthy planting stock but can also facilitate the movement of plants through quarantine. Note that cultures cannot be said to be axenic. Pathogenic organisms may reside within the tissues without displaying any symptoms under prevailing culture conditions only to appear at some later stage. Viruses in particular are not eliminated by routine disinfestation procedures. However, virus indexed plants may be obtained through tissue culture, e.g. by meristem culture.

4. **Plant selection** - It is possible to have a large number of plants or at least growing points within a culture vessel. As mentioned above, there is often some genetic variation within normal cultures. In addition, it is possible to treat cultures in ways which greatly increase the mutation rate. Chemical (mutagenic substances, hormones) or physical (radiation) treatments may be used. Once a genetically diverse population is established the opportunity exists to select out superior genotypes. In addition, because of the controlled environment in culture it is possible to manipulate the conditions to select certain characteristics. For example, cultures may be exposed to a plant pathogen or to increased salt levels until only a few individuals survive. It is likely that the survivors have a higher level of tolerance of the imposed conditions. These may then be multiplied as new, improved stock. Caution is needed though because plants grown out under natural conditions may not display the same level of tolerance; field testing is still needed but the number of plants to be tested can be significantly reduced.

5. **Micro stock plants** - It is being increasingly recognised that the quality and condition of the stock plant or source of propagation material can have dramatic effects on the success of propagation, including tissue culture. Factors such as nutrition, water supply, pathogens, light and temperature may all have an influence. Maintaining extensive stock plants under ideal conditions is generally impractical but such conditions can easily be provided for in vitro cultures. Mother stocks may be maintained in vitro and micro-cuttings taken for rooting either in culture or in a conventional propagation system.

6. **Controlled environment** - Where it is desirable to maintain plant cultures under controlled environment conditions either for cultural requirements, e.g. stock plants discussed above, or perhaps to precondition plants for other purposes, e.g. induce rooting in difficult species, in vitro cultures have an obvious advantage. In vitro cultures are also very convenient for experimental research.

7. **Genetic conservation** - Again the small space required and the greater ease of providing suitable conditions, make in vitro culture a practical way of maintaining stock plants of genotype collections. This could apply to agricultural plants or to rare and endangered species. The limiting factor at present is the need to regularly transfer cultures to fresh medium in order to maintain them. Methods of low-maintenance, long-term storage are being developed. The ultimate is probably cryostatic storage, i.e. deep freezing but the precise conditions for freezing and subsequent thawing without damaging the tissues have to be determined.

8. Tissue culture techniques may be used to obtain hybrids from incompatible species through either embryo or ovule culture.

9. Haploid plants may be obtained through anther culture. Haploid plants have some advantages over diploid material when mutagenesis is used in that recovery of recessive mutation is enhanced. Further, double haploids are homozygous and thus pure breeding.

10. All year round production of plants.

11. Vegetative propagation of difficult to propagate species may be achieved through tissue culture.

Micropropagation

Micropropagation or organ culture commences with an organised part of the plant, most often a bud, and the culture process maintains this organisation whilst directing subsequent growth and development towards multiplication and regeneration of whole new plants (Fig. 9). This is distinct from cultures which involve the production of disorganised tissues such as callus at some stage in the process.

For micropropagation the process involves some or all of the following stages between the collection of the original explant and the supply of new plants at the other end.

1. Selection of suitable plant material.
2. Establishment of aseptic cultures.
3. Multiplication.
4. Elongation.
5. Root formation.
6. Planting out.

At each stage various factors or conditions must be provided to manipulate the plant growth in the desired direction. Most of these factors or conditions are those which regulate natural plant growth and development. Therefore, tissue culture practice should be based on an appreciation of basic plant physiology or biology.

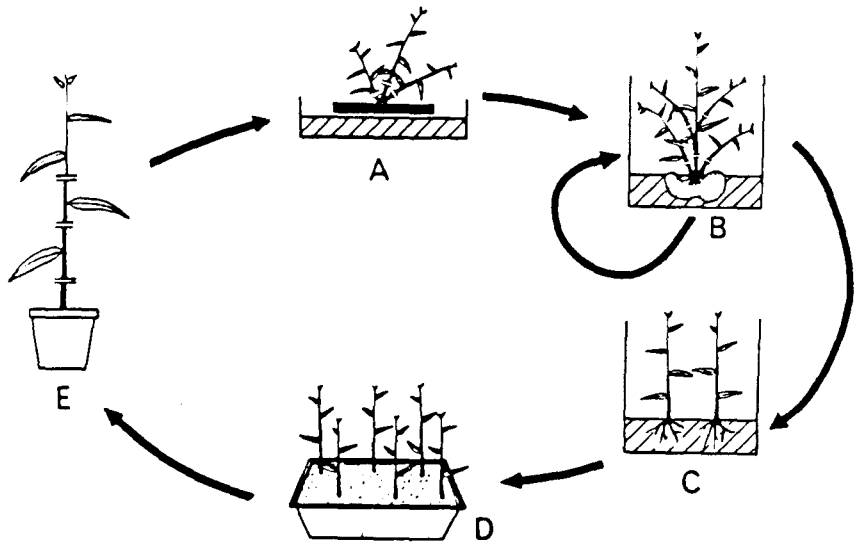


Fig. 9. Diagrammatic summary of the steps involved in the micropropagation through enhanced axillary branching. Single node segments are exised from the parent plant (E) and, after surface-sterilization, planted on a culture medium. In medium containing suitable hormones the axillary bud develops into one or more shoots (A). Individual shoots are excised from these cultures and transferred to a fresh shoot multiplication medium. Within 3-6 weeks each shoot develops several new shoots (B). Individual shoots can be excised again and the shoot multiplication cycle repeated indefinitely (B). When an adequate number of shoots has been produced, some material is kept for further shoot multiplication and some is transferred to rooting medium (C). After a satisfactory root system has developed, the plantlets are transferred to a well-drained potting mix (D) and the material is maintained under high humidity for the first 10-15 days. (From Bhojwani, 1980)

1. Selection of suitable material

1.1 Stock plant selection

1.1.1 Genotype

Apart from the selection of desirable forms of the species, the propensity for tissue culture, particularly adventitious root formation, may also be inheritable. Where possible use material from a range of genetically different parent plants.

1.1.2 Condition of plant

Explants from healthy, vigorous plants are more likely to produce successful cultures (stock plant management, discussed later).

1.1.3 Part of plant

Most commonly a bud or node but may be any other part depending on the species and desired approach.

1.1.4 Size of explant

The smaller the explant the less likelihood of transmitting endogenous infestations or introducing variability due to chimeras where they are present. On the other hand the smaller explant is more likely to be damaged during handling and is more susceptible to failure during initial culture.

1.1.5 Ease of culture

Some species or cultivars are easier to culture than others; generally those which are easy to propagate by conventional cuttings, culture more readily and conversely.

1.1.6 Position on plant

Shoot tips and most recent flushes of growth are best. Avoid material in contact with the soil where infestation is likely.

1.1.7 Avoid diseased tissues

Apart from surface contamination, plant tissues may contain pathogenic organisms. Select only healthy tissues. Actively growing shoot tips tend to have less infestation.

1.1.8 Chimeras

Some plants are prone to localised genetic mutations or chimeras, e.g. coloured patches on leaves, different leaf shapes on a twig. Such genetic traits will be reproduced in culture. However some chimeric traits may be specifically selected as desirable characteristics.

1.1.9 Polyploidy

Normal plant tissues have a paired set of a particular number of chromosomes in their cells. Some individuals or tissues may have additional (polyploidy) or reduced chromosome numbers. This may be due to natural abnormalities or caused by chemical treatments etc. Pollen grains are naturally haploid, i.e. have one set of chromosomes only. These differences may be an advantage or a problem depending on the circumstances.

1.2 Plant growth cycles

1.2.1 Juvenility / maturity

Refers to the 'age' of the tissues in a developmental sense as distinct from chronological age. Mature tissues are produced after a number of growth cycles of the plant and are therefore usually some distance from the original seedling root system, even though they may be from a recent growth flush. Conversely, juvenile tissues are produced from the seedling part of the plant. They may be very old on an established tree but may also be of recent growth, e.g. sprouts produced from a tree stump. Mature tissues have different physiological characteristics which affect their culture requirements.

1.2.2 Vegetative / generative

Developing buds may be vegetative or generative (floral) depending on their position and the growth cycle of the plant. For most purposes vegetative buds are preferable because they will produce a new shoot and therefore multiply the number of growing points. The physiological state of the shoot tissues is different during the plants' flowering period and this can affect the response of vegetative buds collected at that time. It is often recommended that flowering periods be avoided when collecting cuttings or culture material but recent studies with several native species suggest this may not always apply.

1.2.3 Active / dormant

As with flowering, plants and individual buds or tissues go through cycles of growth activity and inactivity (dormancy) and these different states affect the response of the tissues to culture conditions.

1.3 Physiological state

The objective of tissue culture is to control conditions under which the cultured explant is growing in order to manipulate its growth in the direction we require. Growth of any tissue or organ, whether in culture or as part of the intact plant, is ultimately determined by physiological conditions within the tissues. Plant responses to changes in growing conditions must be mediated by physiological changes within the tissues. It follows then that the current physiological conditions within a tissue may affect the end result of changes to the external conditions, i.e. the resulting growth pattern is determined by the nett physiological state as influenced by both internal and external conditions.

In practice this means that the precise conditions needed to elicit a particular growth response in culture will vary depending on the physiological state of the plant material. The physiological state of a plant varies naturally as the plant grows through different stages and as environmental conditions or seasons change. We can control some of these changes either indirectly by controlling the environment, e.g. temperature, light, water supply, nutrient supply or more directly by applying plant growth regulators.

1.3.1 Plant hormones (Plant Growth Regulators)

Many changes in the physiological state of plant tissues are mediated by plant hormones. These chemicals occur in very low concentrations in the tissues and regulate the growth and development of the cells. Hormones, by definition, are produced in one part of the plant and move through the tissues to affect cell activities in another part, e.g. cytokinins produced in the roots may play a key role in stimulating cambial activity in the stem. In this way hormones provide "communication" between different parts of the plant and enable coordinated growth of the whole plant.

In our example of cytokinin, root and shoot growth may be coordinated because when root tip cells are actively growing they produce cytokinins which in turn stimulate growth from shoot cambial tissues and conversely as root growth declines so does the supply of cytokinins to the shoot. Other examples are the production of auxins at shoot tips and the inhibition of lateral bud development as the auxin passes down the stem or the production of abscisic acid in the leaves of some plants under short days and the subsequent induction of dormancy in buds along the shoot. These examples are a simplification of the total picture because most plant responses involve an interaction of hormones and other factors. None-the-less, by manipulating the types and levels of plant hormones in tissue cultures we can directly regulate the pattern of growth in a similar way.

When surveying the literature on plant tissue culture one soon realises that it is hard to make reliable generalisations about the responses to plant hormones or other treatments used. There are some broad generalisations such as:

- auxins usually promote root initiation and callus growth but inhibit root growth and lateral bud growth
- cytokinins promote shoot proliferation and cell division but inhibit root initiation
- gibberellins promote elongation and may overcome dormancy

But there are even exceptions to these. The reality is that so many other factors can influence the responses. Paramount among these is the physiological state of the explant when taken from the parent stock plant. We need to understand how the parent plant's physiological state is influenced by the conditions under which it is grown so that we may either compensate for this or preferably control it to our advantage.

(See Chapter 6 for more information on Plant Growth Regulators.)

1.3.2 Carbohydrate levels

Plants and tissues go through periods of nett carbohydrate (energy) increase and decrease resulting in changes in the level of carbohydrate within the tissues, generally in association with the growth cycles discussed above. They may also affect growth responses. Shoots generally accumulate carbohydrate between periods of shoot or fruit growth and then consume these during the next growth period. Therefore, higher levels of carbohydrate would be expected at either the end or beginning of the growth season.

Since most culture media contain a carbohydrate source, usually sucrose, the level of endogenous carbohydrate may not be critical. On the other hand, where cuttings are being rooted conventionally they are dependent on the internal supply of carbohydrate for their energy supply until an effective leaf surface is re-established. This is the basis of the belief that cuttings (and culture explants) should not be collected during the flowering or fruiting period. Whilst carbohydrate reserves might be highest at the end of the growing season buds may also be entering a state of dormancy at this time therefore the beginning of a new growth flush is preferable.

The carbohydrate balance of the stock plant or that part of the plant which is to be used, can be manipulated by cultural practices such as cincturing or girdling and supplementary lighting or shading (discussed later under stock plant management). The level of sucrose supplied to shoot cultures prior to the excision of shoots for rooting may also be important.

1.3.3 Nutrient status

There are two parameters of nutrient status, actual levels of individual elements available and the balance between them. In culture all essential elements, which would normally be supplied in the soil, must be provided in the medium in suitable proportions. The availability of a nutrient can be affected by the overall balance or by the pH of the medium. The nutritional status of the parent stock plant may also have an effect.

For most tissue culture situations a standard mixture of micro and macronutrients is included and comparison is limited to changes in overall concentrations. The examination of the effects of changes to individual elements alone and in combination with others is too impractical and of a low priority. However, some experiments have demonstrated morphogenic responses to particular elements - particularly micro nutrients which may be important as cofactors in regulatory processes.

1.3.4 Dormancy

Plants do not grow continuously at the same rate. Growth of different parts of the plant is punctuated by periods of little or no growth, usually called dormancy. On any one plant growth of different parts may or may not be synchronised. The most obvious manifestation of this is the loss of leaves and winter dormancy of deciduous trees but even evergreen trees have dormancy; the difference with evergreen plants is that, apart from not losing all their leaves, at any one time some shoots are dormant whilst others are growing.

Dormancy may occur in specific growth centres of the plant including stems, roots and leaves as well as individual buds. Three types or states of dormancy can be discerned based on the origin of the growth inhibition. It can be caused by:

- prevailing environmental conditions, e.g. extremes of temperature (environmental or imposed dormancy),

- inhibition arising from other parts of the plant, e.g. an active terminal growing point may inhibit the growth of lateral buds below **(apical dominance)** or a leaf inhibits the bud in its axil **(correlative inhibition)**,

- conditions which occurred at an earlier time resulting in changes within the tissues which subsequently inhibit growth, e.g. winter dormancy of buds is due to conditions during the previous summer growth period **(rest)**.

Since in tissue culture our aim is usually to encourage rapid growth and development from buds we need to avoid or overcome dormancy. Environmental dormancy can be avoided simply by providing favourable controlled environmental conditions. Correlative inhibition of a bud is overcome by isolating the bud or removal of the terminal growing point and the leaves. The application of cytokinins also has the effect of overcoming apical dominance which is the main cause of shoot multiplication. However rest, by definition, lies within the bud or tissues themselves and cannot be removed by such direct treatments.

The true nature of rest, and indeed other forms of dormancy, is not known. Suggested explanations include both physical and chemical changes within the tissues or surrounding structures of the organ. Some hypotheses assert that rest is caused by the accumulation of inhibitors (e.g. abscisic acid); leaching or neutralisation of these inhibitors may break rest. The application of chemicals including hormones (gibberellins, cytokinins) is sometimes effective. Exposure to chilling temperatures is a natural means of overcoming rest. Wounding of nearby tissues has also been effective.

Rest may be avoided in cultures by taking explants from non-dormant parts of plants, i.e. use actively growing shoot tips or buds just prior to the next growth flush. Stock plants can be managed to prevent the onset of dormancy (discussed later). Leaching the plant material in running water prior to use may also have an effect on rest.

Exposure of cultures to chilling temperatures for several weeks has worked in other cases, particularly seeds or embryos. Bear in mind that rest or other forms of dormancy may be invoked at some stage during the culture programme.

1.3.5 Light

Light has various effects on plant growth apart from providing the energy source for photosynthesis. Conversely the exclusion of light from a plant or a particular tissue can affect its physiology. Carbohydrate levels are reduced under low light intensities or darkness. Changes in endogenous hormone levels or other physiological components can be affected by changes in light intensity, duration or quality (colour). These effects may be on the parent plant or in cultures at any stage.

1.3.6 Water stress

Apart from the transient or eventually permanent wilting which occurs when plants are under water stress, persistent physiological changes may be induced. These in turn may affect other physiological responses discussed above. Abscisic acid has been found to accumulate in some tissues, particularly leaves, under water stress. This may contribute to induction of dormancy or rest. A period of sub-lethal water stress promotes the initiation of flowering in some cases. Wilting and consequent tissue damage are the main concerns in tissue culture particularly during the preparation of initial explants but there has been little investigation of other possible effects.

2. Establishment of aseptic cultures

2.1 Disinfestation

2.1.1 Types of infestation

Explants and cultures may be infested with a variety of micro-organisms including fungi, bacteria, insects or virus. Such organisms are universally present on and within plant tissues. Many are not pathogenic, i.e. they do not cause any harm to the host plant under normal conditions. The dry conditions and presence of competing organisms may keep them under control. However, the conditions in vitro which favour target plant growth, i.e. high levels of nutrients and sucrose, high humidity and warm temperatures, also favour the growth of micro-organisms which often multiply and grow rapidly smothering the explant.

2.1.2 Surface infestations

Infestations may be on the surface of the plant, between the cells or within the plant cells. Surface infestations can be dealt with by various washing and chemical treatments (see Chapter 4 for detailed information). The main limitations are to provide a sufficiently rigorous treatment to eliminate the infestation without damaging the explant tissues. Where the plant surface is covered with hairs or scales care must be taken to ensure penetration of the chemical since contact with the organism is necessary. This is usually achieved by the addition of detergent, agitation or by placing the submerged explants under reduced pressure to remove air bubbles which could harbour micro organisms.

Pre-treatment or management of the stock plants can greatly reduce the initial load of infestation thereby reducing the severity of disinfestation treatment needed and reducing the resultant damage to the tissues. Often a piece of plant is surface sterilized thoroughly then carefully dissected to remove damaged or infested outer tissues and obtain a clean undamaged explant.

2.1.3 Sources of infestation

The initial explant is the major source of infestation but reinfestation is possible at any stage of the culture process. Firstly the medium and all containers and instruments must be sterilized. Thereafter all operations must be carried out under strict hygienic conditions, although not necessarily in a sterile laboratory. The air is a major source of spores and other infesting agents as are the operator's body and clothing.

2.1.4 Endogenous infestations

Organisms living within the plant tissues are more difficult to deal with. These may be controlled to some extent by systemic pesticides and fungicides applied to the stock plant prior to collection of explants or to the cultures themselves. Virus elimination requires more special treatment (discussed later).

It is common to find that infestations appear in cultures after several generations of apparently sterile culture. This may be due to infesting agents which have survived within the tissues until conditions favoured their growth (latent infestations). Alternatively, infestations can be introduced during sub-culturing or may penetrate the closure of the container.

The basic philosophy is to assume that everything which has not been specifically sterilized is a source of reinfestation and all operations are carried out accordingly. Once a culture is infested it is rarely worthwhile trying to save it but some treatments are available for desperate cases (discussed in Chapter 4).

2.2 Virus elimination

Viruses usually reside within the cells of the plant tissue and are transferred to new cells during cell division therefore they are transferred to progeny during vegetative propagation. They may not exhibit any symptoms whilst the plants are growing in culture but show up later when the plant is transferred out.

The main approach to elimination of a virus is the use of heat therapy. Under normal growing conditions a virus will be transferred to new tissues as the shoot tip grows. If the plant can be grown at high temperatures it is possible to slow the replication rate of the virus so that the shoot apex can grow ahead of the infestation. The shoot apex can then be removed and grown free of the virus. It is usually necessary to test the subsequent growth for the presence of the virus to be sure of virus free material (Fig. 10).

Heat treatment can be applied to normal intact plants but the temperature required (e.g. 39°C for 7 days) is often lethal to the plant. Shoots in vitro may be more capable to withstand this treatment.

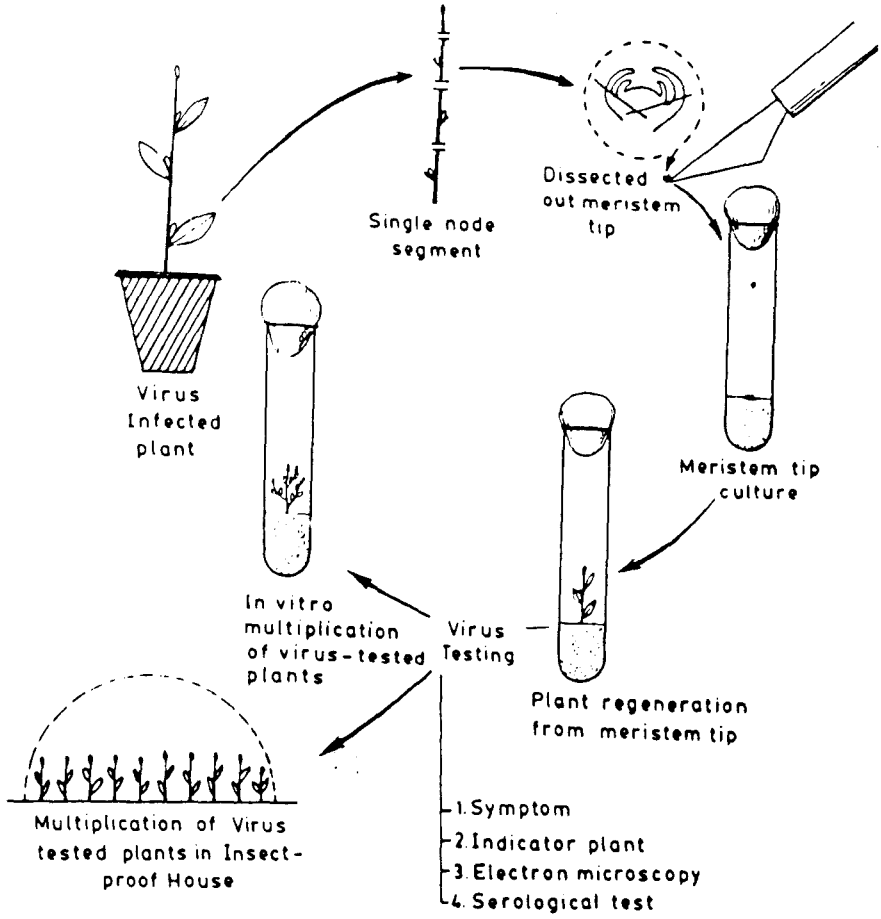


Fig. 10 Diagrammatic summary of production of virus-free plants through shoot tip culture (from Pierik 1987).

2.3 Initial media

It is a general practice to use a basic nutrient medium with sucrose but without hormones for the initial explants. This avoids the waste of complex medium where a proportion of the cultures will be lost due to either infestation or death of the explant as a result of the preparation involved. Most fungal or bacterial infestations will be manifest within the first two weeks.

In some instances a pesticide may be included in the initial medium or sucrose may be omitted to enable the explant to grow away from infested material. This new growth may then be carefully removed for subculturing. It can be difficult to avoid transfer of the infestation especially if sporulation has commenced. However, once placed on a sucrose medium residual infestations will take over. Care must also be taken to avoid contamination of the culture preparation area.

2.4 Exudates

Another type of contamination is exudation from the explant rather than infestation by other organisms. When plant tissues are injured, by cutting or by chemical treatments such as chlorine solution, physiological reactions occur in cells around the wound. Part of this process is the production of biochemical substances either as break down products or synthesized as a defence mechanism. Some simple leaching of substances from the tissues may occur. These chemicals may or may not have a deleterious effect on the growth of the cultures.

Thorough rinsing of the explant material prior to insertion and the avoidance of desiccation can minimize the wound reaction but some species still produce exudates. It may be necessary to transfer the explants to fresh medium frequently over the first weeks of culture to eliminate the exudate. In other cases chemical additives in the medium may be used to counteract or absorb the exudate. Adsorbants include activated charcoal dust (see Appendix 6 for further information) and PVP (polyvinyl porrolidine). Anti-oxidizing agents such as ascorbic acid, citric acid or cysteine may prevent the production of some exudates.

A final soaking of explants in 50°C sterile water for 5 to 15 minutes has been successful in overcoming the production of phenolic exudates in some of the Australian native plants.

Production of a dark exudate in Eucalyptus, may be reduced by placing cultures in darkness for a few days.

Another exudate which is not observable but can have significant effects is ethylene gas. Ethylene is produced naturally in plant tissues and plays a hormonal role in normal plant growth and development. It is often produced as a result of stress, e.g. wounding or desiccation of tissues. Ethylene may accumulate in the culture vessel and affect the explant. Symptoms include leaf senesence and leaf necrosis.

2.5 Cultural conditions

2.5.1 Substrate type

Most micro-propagation is carried out on semi-solid medium where agar, or more recently 'Gelrite', has been included to set the nutrient medium. These gels provide physical support for the explant and increase the aeration of the medium.

Gelrite is a synthetic product which has the advantage of producing a clear gel rather than opaque like agar (an algal extract). This makes observation of infestation or root development easier. It has slightly different physical and chemical properties requiring minor changes in media preparation.

Liquid medium is often used for callus and cell cultures where the tissues must be submerged in the medium to avoid desiccation. Some form of agitation of the medium is needed for aeration and even distribution of the nutrient solution. Vigorous shaking may be used to separate cells or clumps of callus. Explants may also be suspended in liquid medium using filter paper bridges or Sorba rods.*

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* The Sorba Rod Plugs consist of a cold-crimped cellulose paper wrapped with porous cellulose paper, on the model of a cigarette filter. To sterilize the Sorba Rods put the required number in a paper bag and autoclave them for 15 minutes at a temperature of 121°C and pressure of 103 K Pascal.

Sorba Rods are produced by Baumgartner Papiers in Swtizerland. They can be purchased through Jepson, Bolton and Co Ltd, 22 Conduit Place, London W21HS, England. Telephone 01 402 2806, Fax 01 402 3263.

The type of substrate can affect the type of growth and development obtained, e.g. root morphology.

2.5.2 pH of medium

The pH of the medium is most commonly adjusted to 5.5 at the time of preparation. Media pH can affect nutrient solubility, uptake by the culture and setting of agar or have morphogenic effects. One factor usually overlooked is the change in pH of the medium over time and during the process of autoclaving.

There may also be an interaction between pH and other factors in the medium. Our work has shown that pH can have a marked effect on the response of explants to applied hormones, particularly in root formation.

2.5.3 Environment

The main environmental factors for cultures are light and temperature since moisture levels are maintained within the closed containers. Most cultures are kept at room temperature, i.e. 20-25°C. Light is usually supplied by fluorescent tubes, either white plus supplementary incandescent globes or growlux tubes, giving approximately 50μ $EM^{-2}S^{-1}$ irradiance at culture level. This relatively low irradiance level is adequate for normal morphological responses but insufficient for photosynthesis which is not essential where sucrose is supplied in the medium. The photoperiod or daylength is typically 12-16 hrs, sometimes 24 hours.

A heavily shaded area in a glasshouse or adjacent a window in a room could be adequate for small scale routine work.

2.6 Observation and transfer

A high percentage of initial cultures may be infested particularly where explants have been obtained from field grown plants. Other explants may be damaged by the preparation and disinfestation process. These effects will usually be evident within the first two weeks in culture. Surviving explants can then be transferred to more complex culture medium. If the production of exudates is a problem, several transfers on to fresh basal medium may be required during this establishment period.

Some bud cultures may produce shoot elongation growth during this early stage and these shoots can be subdivided at the time of transfer to new medium.

3. Multiplication

Once aseptic cultures have been established the objective is to induce shoot multiplication. In some species explants may produce roots during this early stage on simple medium, i.e. they perform as micro cuttings. This is of little value since the aim is to produce many plants not single rooted plants from each explant. Other species produce multiple shoots without further treatment. In these cases the need for more complex multiplication media will depend on the level of multiplication obtained or desired.

3.1 Types of multiplication

Shoot multiplication may be obtained by several paths.

- The existing shoot tip or bud may elongate to give new nodes and internodes which can be then subdivided (Fig. 11).

- Lateral buds present on the explant may produce shoots which themselves have further buds along them. Often these lateral buds are hardly visible to the naked eye but most leaf axils contain numerous primordial buds (Fig. 11).

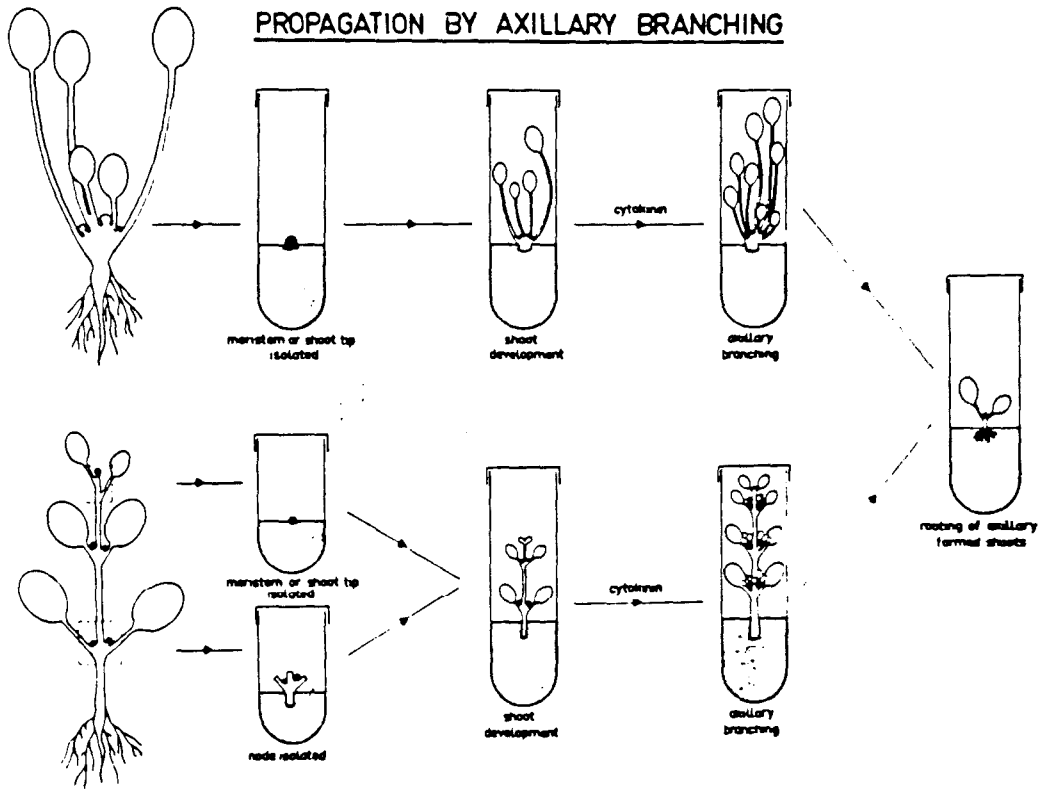

Fig. 11 Schematic representation of the axillary bud method of vegetatively propagating plants. Upper row: as applied to rosette plants. Bottom row: as applied to plants which elongate. (From Pierik 1987)

- Adventitious shoot development. In many species, plant organs, e.g. roots, shoots, or bulbs may be induced to form on tissue which normally do not produce these organs. Such adventitious organogenesis has more potential than the induction of axillary buds for mass clonal propagation of plants. A single leaf, for example, may produce thousands of buds or shoots each genetically identical to the explant.

- Somatic embryogenesis. The greatest potential of clonal multiplication is through somatic embryogenesis where, technically, a single isolated cell can produce first an embryo then a complete plant. Somatic embryogenesis may occur in suspension cultures or occasionally in callus. Induction of embryogenesis requires exposure to an auxin, often 2,4-D followed by a reduction in auxin level. Embryo induction also requires a source of reduced nitrogen.

3.2 Controlling factors

Existing buds may not grow out under normal growing conditions because they are inhibited (i.e. held dormant) by the subtending leaf or by apical dominance (inhibition from the shoot apex or distal buds). Removal of the shoot tip (pinching) as explants are prepared may overcome apical dominance but often hormone treatments are used. The production of multiple shoots on cytokinin rich media is often due to release of existing buds from this apical dominance.

Rest may also prevent existing buds from growing. This is not usually the case where explants are taken from growing shoots on the stock plant. Chilling treatments, gibberellin or ethylene applications or long light periods may overcome rest. Research with apple trees has also shown that wounding (cutting) the shoot distal to a bud can break rest therefore the process of cutting shoots into internodes may overcome this form of dormancy.

The production of shoot multiplication from existing buds is the simplest and safest method because it does not involve differentiation from other tissues with the inherent risk of somatic mutation discussed earlier.

Multiplying cultures may be repeatedly subdivided to produce many shoots; referred to as **bulking-up**. Sometimes physiological or morphological anomalies **(Vitrification)** may occur when cultures are sub-divided through numerous cycles. This may result in loss of vigour in the cultures or an increase in somatic mutation. For this reason it is a good practice to maintain mother stock cultures which are infrequently subcultured and sections of this are taken for the mass production cycle. Such production cultures are discarded at regular intervals and new ones commenced from the original mother stock culture. On the other hand, numerous sub-culture cycles may result in an increase in rootability, discussed in this Chapter - Section 5.

3.3 Multiplication rate

The number of plants produced from each explant differs for different culture conditions and for different species. In the case of strawberry 1.5×10^7 plants can be produced in a year from a single explant. Table 2.1 provides a guide to the number of plants which could be produced from one explant in a year based on different multiplication rates, and monthly passages. While the figures provide an indication of the potential which this technique has for providing large numbers of plants, it must be emphasized that these are theoretical figures and are most unlikely to be achieved in practice.

Table 2.1 Theoretical multiplication rates based on passage to new medium each month.

Multiplication rate/month	Thousands of plants per year
2.0	4
3.0	531
3.5	3,379
4.0	16,777
4.5	68,953
5.0	244,140

4. Elongation

Having obtained shoot multiplication, it may be necessary to provide particular conditions for shoot elongation to get shoots long enough to be handled. Often transferring cultures to a hormone free medium after the multiplication stage is sufficient to promote shoot growth. The application of GA may induce elongation growth.

5. Root formation

Once a crop of shoots has been produced the next stage of root initiation may be carried out in vitro or the shoots harvested and treated as micro cuttings under non-sterile conditions. Either way cultures are usually dissected into individual shoots at this stage. Some shoots may be recycled for further multiplication.

For practical production of easy-to-root species, the rooting of the micro cuttings out of culture is more economic. It avoids the preparation of additional medium and the need for aseptic working routines. Where the species is more difficult to root the extra labour of in vitro culture is often worthwhile.

If the micro cuttings are to be rooted out of culture care must be taken to provide suitable conditions. High humidity is necessary to prevent desiccation of the soft shoots. Micro cuttings may be treated with rooting hormones (auxin powder or liquid dip) just like conventional cuttings.

Another advantage of rooting micro cuttings out of culture may be the type of roots produced. Roots which develop in agar or liquid medium often have a morphology adapted to water or nutrient uptake from such substrates rather from a soil matrix. These roots may be non-functional in soil and therefore have to be replaced if the plantlet is to survive. It could be quicker to root micro cuttings directly in soil.

With some species adventitious roots form readily even during the multiplication stage whilst others produce roots when simply transferred to cytokinin-free medium. However many species require particular conditions usually involving auxin in the medium. There is considerable specificity in responses to types, levels and combinations of auxins and other culture conditions.

Cytokinins generally inhibit root formation (there are exceptions) and so are not included in rooting media. There may be a carry-over effect of cytokinin treatments during the previous multiplication stage so an intermediate step on hormone-free medium may be beneficial.

As with other culture stages, there is often an interaction between factors including pH, light, nutrients (particularly calcium) and the hormone treatment. Apart from weaning off from cytokinin treatments, preconditioning of the shoots before excision and transfer to rooting medium may promote rooting. Preconditioning may include etiolation or chilling treatments. Even where micro cuttings are to be harvested and treated conventionally, pretreatment of shoot cultures may promote rooting later.

Whilst auxins may promote root initiation they can inhibit subsequent root growth therefore a transient exposure to the hormone may be more effective. There may be no visible sign of rooting on the initiation medium but they emerge on transfer to an auxin free medium.

Rooting of woody species is usually more difficult than herbaceous species. For woody plants, cultures derived from seedlings (Juvenile Phase) may root more readily. A similar effect may be obtained where cultures have passed through numerous multiplication cycles or subdivisions. This is attributed to rejuvenation of the shoot material. Other juvenile characteristics may be seen too, e.g. a different leaf shape. The physiological basis of this effect is not known.

6. Planting out, deflasking or acclimatization

Whether shoots are being harvested as micro cuttings or rooted plants are being transferred to soil the plants are subject to a marked change in environment and are liable to be severely stressed unless adequate precautions are taken. This is often the critical stage in the overall tissue culture cycle where losses can be high.

The in vitro environment includes high humidity, freedom from pathogens, optimal nutrient supply, low light intensity and a supply of sucrose plus a liquid or gel substrate. The plants produced are adapted to these conditions. When exposed to the outside environment the small plants must adapt and this generally occurs as new growth is produced rather than modification of existing organs. If the transition is too abrupt plants will collapse.

Leaves produced under high humidity/low transpiration potential, tend to have thinner cuticular wax layers and a more open mesophyl tissue. Under low light intensity they may have reduced chlorophyll levels. It is also thought that the photosynthetic processes are inhibited by the presence of sucrose in the medium. As discussed above roots also adapt to their immediate environment. Gradual exposure to normal conditions leads to progressive morphological and physiological adaption, i.e. **hardening-off**. This gradual effect can be achieved by modifying the culture conditions prior to transplanting to precondition the plant or by carefully controlling the environment for a period after transplanting.

The relative humidity in vitro may be reduced either by loosening the vessel enclosure or by increasing the agar concentration. A reduction in sucrose level and increase in light intensity during the weeks preceding transplanting may activate chlorophyll synthesis and photosynthetic activity. Similar changes may occur to the root system. In addition, root morphology may be affected by the type of hormone used or the pH of the medium.

The conditions required for hardening plants when transferred out of culture are similar to those necessary for very soft cuttings. They must be given high humidity and low light intensity initially with a progressive reduction in humidity and an increase in light level over the following couple of weeks. The medium into which they are transplanted should retain moisture but have adequate drainage. The soil and surrounding environment need not be sterile but a high level of hygiene is important under the very humid conditions. For small batches a box with a clear lid which can be gradually removed is adequate. Place the box in the shade at first and then progressively move it into normal light to suit the species (see Appendix 13 for further details).

CHAPTER 3

OTHER TYPES OF CULTURE

Tissue culture techniques other than micropropagation generally require much more sophisticated operations but may offer greater benefits in the future as the techniques are refined and some problems are overcome. Some are already valuable scientific tools for disease elimination and plant improvement, including **'genetic engineering'**.

3.1 Meristem Culture

This term is often used loosely to refer to very small shoot apices dissected from terminal or lateral buds. Strictly speaking it refers to the microscopic apical dome with only the smallest leaf primordia evident, usually less than 2 mm across.

The advantage of using shoot meristems is that they are most likely to be free of internal pathogens (e.g. for virus eradication) and minimize the occurrence of chimeric variation in the cultures. Their major disadvantage is that they are very susceptible to damage and require delicate dissection under a microscope. The general cultural requirements are the same as for larger explants but initial losses are likely to be high.

3.2 Callus Culture

For micro-propagation it is usually desirable to avoid callus formation because it introduces variability and, particularly at the rooting zone, causes a discontinuity with the main vascular system. Sometimes explants produce callus rather than new shoot growth particularly where high levels of hormones are applied. In other cases callus may be induced intentionally because of its potential for mass production of new plantlets. The limiting factors are the difficulty of inducing the initiation of new shoot apices, especially in woody species and the high incidence of somatic mutation.

Much of the early work in tissue culture involved callus culture of tobacco, carrot, petunia etc, all herbaceous plants. With these the general approach was to manipulate the balance of cytokinins and auxins applied to regulate the growth pattern for shoot or root production. This lead the way to the development of the commonly used media but multiplication from existing shoot meristems is the predominant path in current practice.

The greatest potential use of callus culture is where the callus cells can be separated and induced to differentiate into somatic embryos. Morphologically, these embryos resemble those found in seeds but unlike seed embryos they are genetically identical to the parent plant, i.e. sexual segregation of genetic material is not involved. Since a millilitre of callus contains thousands of cells, each capable of forming an embryo, the multiplication rate can be tremendous.

Callus culture lends itself readily to automated production because it can be done in liquid medium and the embryos develop as separate individuals making handling simpler. One approach with considerable attraction is the production of artificial (somatic) seeds by enveloping the embryos in a protective covering. They might then be handled like conventional seed. Thus we could have clonal seed of selected plants on a scale suitable for field crops (Fig. 12).

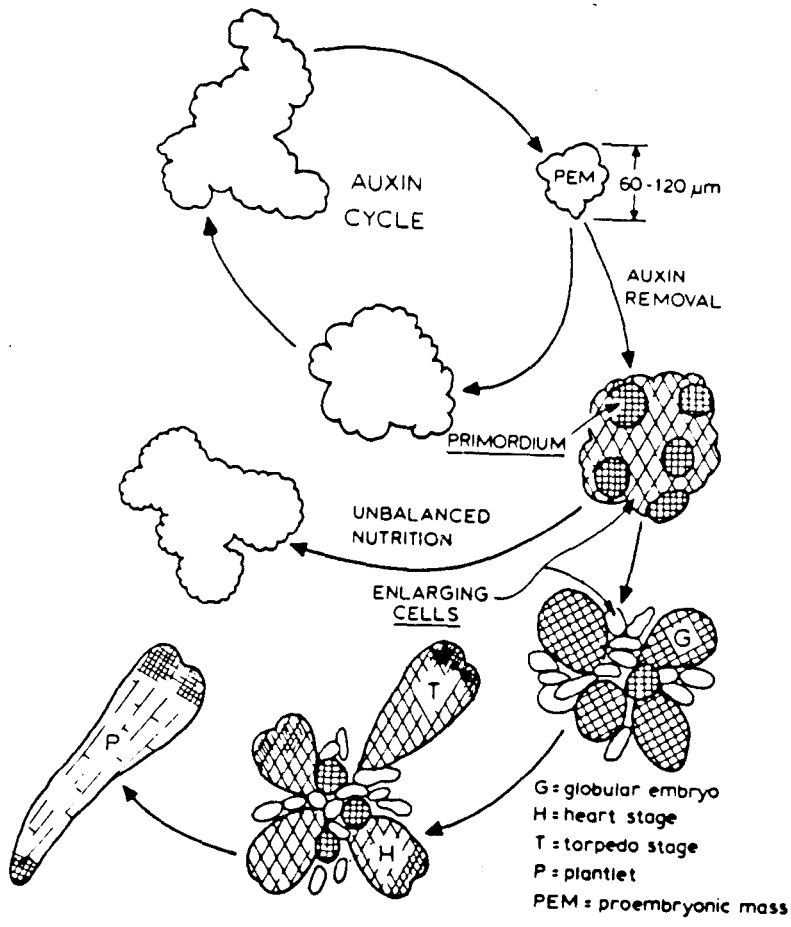

Fig. 12 Diagrammatic representation of in vitor embryogenesis in suspension cultures of wild carrot. (From Wetherell, 1978)

3.3 Cell Suspension

This is essentially a product of callus culture, i.e. callus usually refers to a mass of undifferentiated cells, once these are separated in liquid culture it becomes a cell suspension.

Cell suspension culture may be used to produce a product directly from these cells without regenerating new whole plants. The product may include the cell mass or be a chemical extract. Such cultures are similar to those of micro-organisms. The cells used may be genetically engineered to increase the synthesis of a particular substance (Fig. 13).

3.4 Protoplast Culture

This is the next step beyond cell suspension culture where the cell walls of suspended cells are removed using enzymes to digest the cellulose to leave isolated protoplasts, i.e. the cell contents surrounded by a semipermeable membrane. With the cell wall removed it is possible to insert or remove foreign materials, including the basic genetic materials DNA and RNA, or to fuse together cells from entirely different species.

Whilst it may be technically possible to manipulate the genetic composition of cells the application of these techniques is limited by our scant knowledge of the genetic code of most plants. We will need to know the DNA sequences and which genomes regulate the characteristics we wish to control. This is further complicated by the involvement of multiple genes in many aspects of plant growth. None the less the engineering of simple gene characteristics is possible.

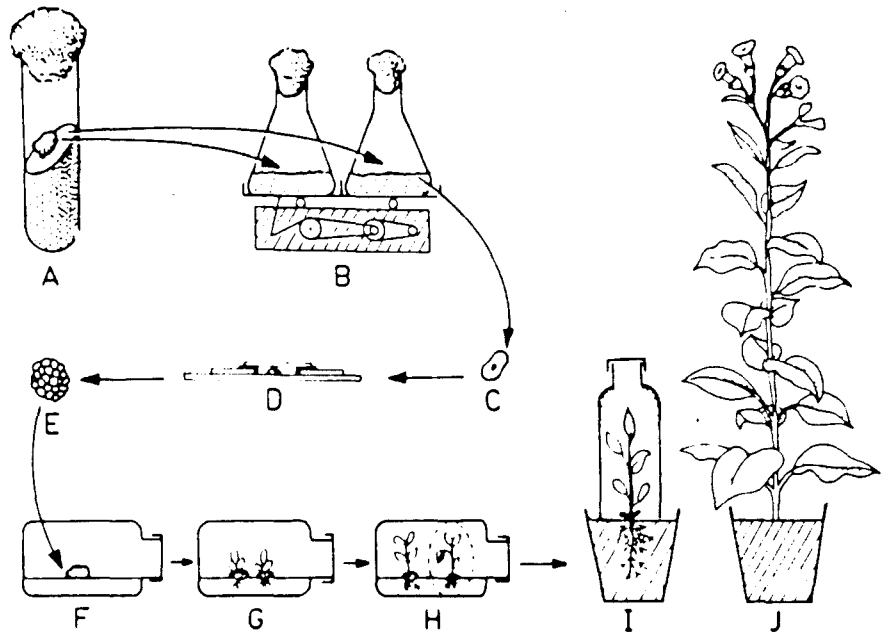

Fig. 13 Development of a tobacco plant from a single cell. A callus is raised from a small piece of tissue excised from the pith (A). By transferring it to a liquid medium and shaking the culture flasks (B) the callus is dissociated into single cells. A cell (C) is mechanically removed from the flask and placed in a drop of culture medium in a micro-chamber (D). A small tissue (E) derived from the cell through repeated divisions is then transferred to a semi-solid medium where it grows into a large callus (F), and eventually differentiates plants (G,H). When transferred to soil (I,J) these plants grow to maturity, flower and set seeds. (From the work of Vasil and Hildebrandt, 1965.)

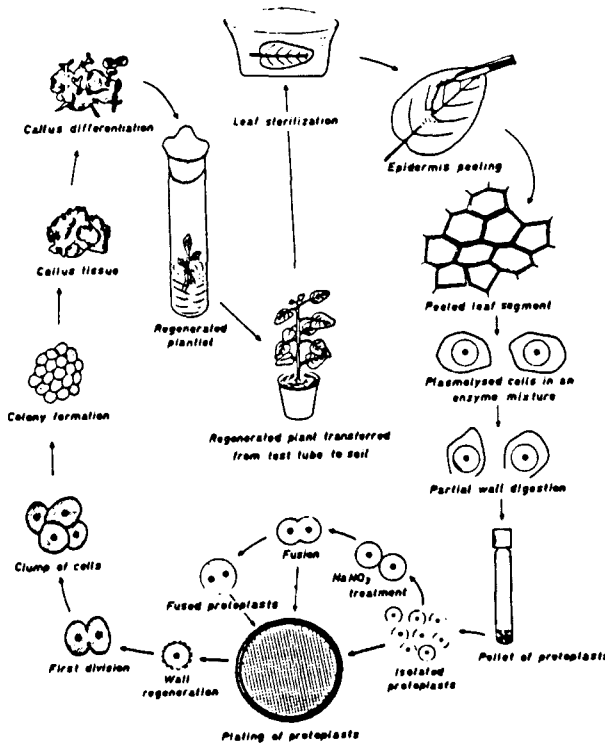

Fig. 14 Schematic illustration of isolation, fusion and culture of leaf protoplasts, followed by callus formation and regeneration of plants. (From Bajaj 1974).

Perhaps of more immediate use will be the fusion of entire protoplasts of otherwise incompatible species. This will enable the combination of attributes from quite diverse species. One topical project is the incorporation of nitrogen fixing attributes from legumes into cereals (Fig. 14).

3.5 Anther and Pollen Culture

The production of callus and somatic embryos from pollen and anther cultures has already been achieved for a number of species. A major attraction here is the production of haploid embryos, i.e. those having only one set of the normal chromosome pair. These arise from gametophytic tissues within the anther. When the chromosome number is subsequently doubled such as by colchicine treatment, the new plants have identical chromosome pairs, i.e. they are homozygous and therefore will breed true to type.

3.6 Immature Embryo Culture

The culture of immature embryos extracted from seeds has two main applications. In some instances incompatibility between species or cultivars arises after embryo formation resulting in embryo abortion. Such embryos may be extracted whilst immature and before abortion and grown on in culture. The other instance is where mature seed is not obtainable for a species perhaps because seed is shed and lost suddenly when ripe or developing seed is attacked by insects before maturation. Embryo culture is relatively simple in that the embryo is a miniature complete plant and therefore no de novo differentiation of shoots or roots is needed.

3.7 Fern Spore Culture

The culture of fern spores in vitro is not strictly tissue culture but rather a means of growing spore under controlled, sterile conditions. It provides ideal growing conditions but the growth pattern is the same as under natural conditions. This is distinct from cultures derived from other parts of the fern plant involving other techniques already discussed.

CHAPTER 4

FACILITIES AND TECHNIQUES FOR PLANT TISSUE CULTURE

4.1 Facilities

To meet the needs of plant tissue culture a laboratory must have adequate space for the performance of several functions. It must provide:

(1) facilities for media preparation, sterilization, chemical storage, preparation of plants for aseptic techniques,

(2) transfer or laminar flow cabinets for aseptic manipulation of plant material,

(3) environmental control rooms or growth cabinets to allow growth of plants under controlled conditions,

(4) microscope facilities for examination and evaluation of cultures preferably equipped with photomicrographic equipment for recording of culture progress.

The ideal organization will allow a separate room for each of the following functions:

media preparation, aseptic procedures, incubation of cultures and general laboratory operations.

If one has the opportunity to plan a tissue culture laboratory in advance, the component facilities should be arranged as a production line. The area designated to washing and storage of glassware should lead to the facilities for oven sterilization and media preparation. Materials should then move from autoclave to the aseptic transfer facility. After the aseptic operations, the cultures should be transferred to growth rooms or growth cabinets. Ideally the growth cabinets and growth rooms should be in close proximity to the microscope laboratory.

4.2 Dishwashing procedure

All discarded cultures, as well as contaminated ones, should be autoclaved to liquefy the agar and kill any micro organisms that may be present. The culture vessels should then be emptied, rinsed and soaked in a detergent bath over night. The glassware can then be scrubbed with a brush and rinsed three times with tap water followed by three rinses in distilled water.

All new culture vessels and any other new glassware used in tissue culture laboratories should be washed thoroughly prior to use.

The glassware should be stored in clean cabinets after drying.

4.3 Media preparation

A sensitive balance to weigh small quantities of plant growth regulators and vitamins as well as a top loading balance for weighing gelling agent and carbohydrates are required. The media reagents should be housed conveniently near the balances. A refrigerator in the media room is necessary for storing stock solutions and chemicals which degrade at room temperature. A combination of hot plate and magnetic stirrer for dissolving reagents would save a great deal of time. A pH meter is required for adjusting the pH of media. Both single and high purity double distilled water are required in the media preparation room. Media should be sterilized either in an autoclave or a pressure cooker. Depending on the volume of medium and size of a culture vessel the autoclaving time* varies between 15 to 40 minutes at 121°C and pressure of 103 K Pascal (see Table 4.1: extracted from Biondi & Thorpe 1981).

Table 4.1 Minimum times required to sterilize different volumes of media by autoclaving at 121°C and 103 K Pa.

Volume of liquid in container (mL)	Sterilization time (min)
20-50	15
75	20
250-500	25
1000	30
1500	35
2000	40

It is important to note that certain plant growth regulators, vitamins and antibiotics are affected by heat and hence need to be filter sterilized. Filter sterilization or membrane filtration involves passing a solution preferably made up with sterile water in a laminar flow cabinet) through an autoclaved or pre-sterilized membrane of pore size 0.45 μm or 0.22 μm under reduced pressure into a sterile vessel. The correct quantity of sterile solution can then be dispensed to culture medium which has been autoclaved previously and cooled down to 40°C in a hot water bath controlled at that temperature.

The laminar flow cabinet is generally surface sterilized with 70% (v/v) alcohol. Although acidified alcohol (70% v/v, pH 2.0) may be more effective as a disinfectant, it is not commonly used because of its corrosive effect on metal surfaces. All instruments can be dipped in a solution of 70-80% (v/v) ethanol and be flamed on a spirit lamp. For safety purposes it is best to put the beaker containing alcohol for flaming inside a holder with a heavy base. This will stop the accidental knocking of the alcohol and hence a fire inside a laminar flow cabinet. Avoid prolonged heating of the instruments after evaporation of alcohol. As a general rule we dispose of alcohol left in the beaker after a culture run or use it for swabbing work surfaces.

4.4 Disinfestation of plant material

The biggest problem facing a tissue culturist is microbial contamination of cultures (both bacteria and fungi). Two ways can be employed to reduce contamination of cultures.

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* In our laboratory we routinely autoclave media, irrespective of their volume, only for 15 minutes at 121°C and pressure of 103 KPa. This seems to be adequate for complete eradication of all the micro organisms which may be present in the jars or media at the time of autoclaving.

4.4.1 Physical methods

Physical methods to overcome the microbial contamination problem are aimed at reducing the size of the microbial population. These methods include:

1. Exposure of the stock plants to dry conditions for 3 to 4 weeks prior to the start of the tissue culture work. The plant is kept well watered and well fertilized and treated with pesticides and fungicides if required. Overhead watering is avoided. The following table shows the population of micro organisms in tomato flowers kept under different conditions.

Table 4.2 Average number of micro organisms per Tomato Flower (de Fossard 1976).

Source of flowers	Non-disinfested	Disinfested*	Contamination % in culture after disinfestation
Field	1,300,000	92,000	100
Glasshouse	85,520	1,600	60
Phytotron	90	43	30

* Plants were disinfested with chlorinated lime 5% (w/v) treated for 20 minutes.

2. At the start of tissue culture work the plant is thoroughly cleaned, and parts which will not be cultured are discarded. The cleaning can involve washing, scrubbing and even scraping to remove all soil particles and dead leaves. It also involves removing most of each leaf because, in most cases, these do not form part of the culture.

3. The cleaned up and trimmed up material is then given a wash under running tap water for 20 minutes, to several hours, depending on the source of plant material. This literally washes millions of microbes down the drain.

4.4.2 Chemical Methods

This may be accomplished with an aqueous solution of sodium hypochlorite (NaOCl). Most laboratories use a household bleach such as White King. This commercial product contains 4% available chlorine. 25 ml of White King made up to 100 m L with water gives a concentration of 1% available chlorine. Because of complete dissociation, hypochlorite has relatively little activity at pH over 8.0 and it is much more effective if the solution is buffered at about pH 6.0 (Behagel, 1971). To improve the success rate with chlorine the following steps may also be incorporated:

(1) Add a detergent to the chlorine solution, e.g. a few drops of Tween 20 or Triton.

(2) Apply reduced pressure during the chlorine treatment. This can be done with a vacuum desiccator fitted to a water or another type of pump.

(3) Agitate the chlorine solution either manually or with a shaker during the disinfestation period. All above techniques will improve the contact of the plant with chlorine solution.

The treatment period with chlorine solutions varies with the type and sensitivity of the plant material.

4.4.3 Endogenous contaminants - Use of antibiotics

Whilst chlorine solution can destroy the external micro organisms it can do nothing for the endogenous or internal micro organisms within the plant tissue. Some laboratories resort to use of antibiotics. Although antibiotics are routinely used in animal tissue culture, its use in plant tissue culture has not been very successful. No known antibiotic is effective against all micro organisms which might cause contamination. Antibiotics and their degradation products are metabolized by plant tissue with unpredictable results. In our view use of antibiotics should be avoided as far as possible.

It is dangerous to develop a micro-propagation system that is based upon the incorporation of antibiotics in the medium for the following reasons:

(1) plants are produced which are endogenously contaminated,

(2) by the use of specific antibiotics one can select for mutant strains which will no longer be controlled by this specific product,

(3) a non-pathogenic contaminant can become pathogenic, either by mutation or physically. Indeed, non-pathogenic bacteria without competition from other bacteria can become overwhelming,

(4) Camouflage of the problems in vitro can be responsible for severe difficulties later on in culture (e.g. bacterial wilt or spots),

(5) bacterial contamination can cause difficulties in the end stages of a micropropagated process: e.g. difficulty to produce roots on contaminated shoots.

4.4.4 Recovering contaminated cultures

The infested cultures may be saved using the following simple method.

(1) Open the containers containing infested cultures and fill them to the brim with a solution of 0.5-1% w/v sodium hypochlorite.

(2) Allow to stand in the chlorine solution for 10-50 minutes depending on the severity of infestation or the sensitivity of the plant material.

(3) Remove the cultures from the chlorine solution, trim the base and remove excess leaves.

(4) Transfer to fresh culture medium.

It is optional whether the explants should be rinsed with sterile water or by passing them through a series of diluted sodium hypochlorite, e.g. 1% → 0.5% → 0.25% → 0.01% and planted without a final rinse in sterile water. This means the piece of plant finally placed onto sterile medium carries with it some chlorine. This is particularly useful in heavily contaminated cultures, bearing in mind that only non-chlorine-sensitive plants could be treated in this manner.

With this method of recovering infested cultures the majority of leaves may become badly bleached. These cultures will soon recover and grow successfully. We have had a recovery success of 50% with some badly infested *Melaleuca alternifolia* cultures.

4.5 Sterilization of glassware and instruments

Culture vessels should pose little potential for contamination since there are usually autoclaved with the medium. Other glassware or culture vessels can be sterilized by several methods, e.g. exposure to U.V. radiation, the use of surface disinfecting fluids or more easily by autoclaving or by the use of a dry heat in an oven at 180°C for a minimum of three hours. Plasticware such as polypropylene or polycarbonate should be sterilized in the autoclave as they will not withstand dry heat at 180°C. Plastic containers can be reused numerous times; as they withstand repeated autoclaving but eventually become brittle.

For dry heat sterilization, instruments such as scalpels, scissors and forceps, petri dishes, beakers etc, can be wrapped first, to prevent contamination during the cooling period.

Boxes of tissue can also be autoclaved by first removing the plastic cover from around the mouth of the box and then wrapped in brown paper or alfoil to be autoclaved. Any paper autoclaved in this manner should be left to dry either in an oven set at 60-70°C or inside the laminar flow cabinet before use.

All sterilized material should be kept wrapped and stored in a clean cupboard. They are sterile as long as the contaminating organisms do not have access to them.

4.6 Sterile technique - the manipulation of plant materials

The primary sources of contamination are the fungal spores and bacteria which form a natural part of the atmosphere. One must assume that contaminating agents are everywhere, e.g. clothing, skin, hair and the breath of the operator, plant tissue, instruments, exterior of the culture vessels, the working surface; to name just a few.

The sterile air inside a laminar flow cabinet allows us to freely open culture tubes and make sterile transfers.

Instruments can be sterilized by dipping them in 70-80% alcohol followed by flaming using a Bunsen burner or a spirit lamp. There is a special Bunsen burner that saves gas and minimizes the danger of an open flame, a Touch-o-matic. A pinpoint pilot flame burns continuously to light the higher flame which is triggered by resting one's hand on a disc while flaming an instrument.

Use of bleach can be an alternative to sterilizing instruments with alcohol. A dilute chlorine solution (0.1-0.25% available chlorine) can be used for this purpose. Instruments must be stainless steel or they will rust rapidly in bleach. When using bleach, protect your skin by wearing rubber gloves. Another option is an electrical sterilizer such as a Bacti-Cinerator II Sterilizer (Fig. 15). Instruments can be sterilized rapidly (5-15 seconds) at a temperature of 900°C as the instruments are inserted into the red-hot, hollow cone of the cylinder. Another method is the hot bead sterilizer.

Some important points to bear in mind when working in a transfer cabinet.

(1) Avoid any obstruction of the laminar air flow because it will change the air flow pattern and so invite contamination i.e. place nothing between the work area and the source of air flow.

(2) Work at arms length, as far back as practical in the hood.

(3) Keep the right hand on the right hand side and the left hand on the left-hand side of the transfer hood work area. Avoid crossing over as far as possible.

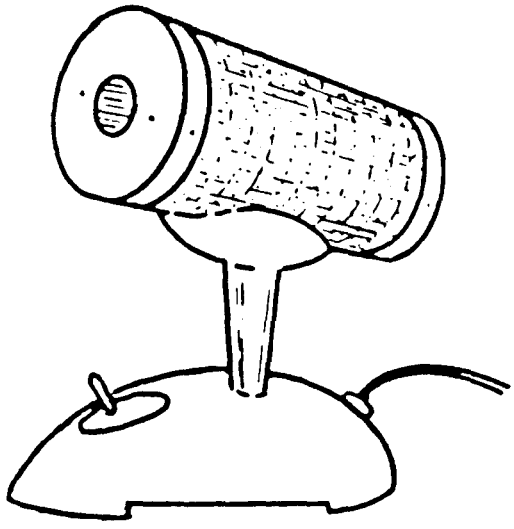

Fig. 15 Bacti-Cinerator II Sterilizer.

In summary the following steps should be taken when attempting to do plant tissue culture work:

(1) Spray or swab the interior of the laminar flow cabinet with 70% ethyl or isopropyl alcohol prior to switching on the cabinet. (It is important that 70% alcohol be used. 95% or absolute alcohol can harbour bacteria and fungal spores without killing them.)

(2) Switch on the cabinet. If you plan to use the UV lamp (see Appendix 4 for further information) make sure this is switched off before plant material is placed in the cabinet.

(3) Spray all vessels and materials with 70% ethanol before placing them in the cabinet.

(4) Wash your hands and arms with soap and water and then swab with 70% ethanol before carrying out plant manipulations. It is important to note that ethanol has no residual effect; therefore it is advisable to use Hexifoam* instead.

(5) If using a flame exercise extreme caution.

(6) Arrange your work area within the cabinet so that crossing over is minimized or eliminated altogether.

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* Hexifoam is a skin disinfectant. The active ingredients are 0.5% W/W Chlorohexidine Acetate and 60% W/W ethanol. Residual effect of Hexifoam is 2 hours. Hexifoam can be purchased from Soltec Research Pty Ltd, 8 Macro Court, Rowville Victoria 3178, phone (03) 763 0022.

(7) When using the Bacti-Cinerator warm it up for 10 minutes to achieve a strong red glow. Sterilize instruments as follows:

Fine scalpels	6 seconds
Fine forceps	8 seconds
Long scalpels	8 seconds
Heavy forceps	10 seconds

Make sure the incinerator has recovered its strong red glow before sterilizing the next instrument. Do not overheat instruments. Resterilize frequently.

(8) Allow instruments to cool on a sterile rack.

(9) If plant material falls onto the floor of the cabinet, assume it is contaminated and discard it.

(10) After completion of your transfer, switch the cabinet off, spray it with 70% ethanol and replace the front panel.

4.7 The environment of the culture room

It is very important that the culture room or growth room be as clean as possible. The use of HEPA filtered air and the maintenance of a positive pressure in the culture room will avoid most problems. When the culture room is designed, direct entry from a dirty area is to be avoided. Entry through a small room which serves as a buffer is desirable. This room may have a sponge foot bath or sticky mat to remove carriers of organisms such as soil particles, fluff etc., from the operators feet. In some cases fans to provide strong positive pressure in the entry room and activated by opening the outer door, are used as an additional precaution. It is wise to limit access to the culture room to a few people. There is little doubt that increased traffic into the culture room increases the risks of contamination particularly from mites. In some culture rooms, visitors must use overshoes before entry.

The clothes of the laboratory staff should always be clean. The need for additional precautions such as caps, face masks and gloves will be determined by the environment in which the work is being done. Hot, humid or dusty environments have a much higher risk of contamination than environments which are cool with low humidity and little dust. Many laboratories use air conditioning to control the temperature of the laboratory as well as the environment in the culture room. It is necessary to select air conditioning systems which causes as little air movement as possible since the transfer of micro organisms via air currents is an important source of contaminants.

CHAPTER 5

TISSUE CULTURE MEDIA

One of the difficulties in plant tissue culture is that the nutritional requirements for optimum growth is very different for most species, so no single medium can be recommended for universal use. Intensive work in tissue culture during the past 35 years has developed many media, a few of which have quite widespread use in plant tissue culture today. These media are compared in a table in Appendix 2. The medium may be chemically defined, meaning only known nutrients are included in the medium, or the medium may be undefined meaning that it contains some complex additives such as coconut milk or orange juice which contain unknown growth factors.

5.1 The Composition of Tissue Culture Media

5.1.1 Inorganic Nutrients

There are 12 mineral nutrients which are essential to the growth of plants and several others are reported to affect plants in vitro. For normal growth in tissue culture these essential elements must be included in culture media. A comparison of the five media in Appendix 2 shows that these essential elements are included in each medium but the concentration differs as may the form in which the element is added.

5.1.2 Organic Nutrients

Plants growing under normal conditions are true autotrophs and are able to synthesise all of their organic requirements. Although plants *in vitro* are capable of synthesizing these compounds, it is believed that they produce insufficient quantities of some of the vitamins for healthy growth and one or more vitamins have to be added to the medium. Of these thiamine is essential and nicotinic acid, pyridoxine and inositol are also usually added.

In addition to these organic nutrients, complex mixtures are often added, these include yeast extract, casein hydrolysate, coconut milk, orange juice, banana tissue and many others. The addition of one or more of these complex mixtures results in an undefined medium. With sufficient research it should be possible to replace the complex mixture with a single substance, perhaps an additional vitamin or an amino acid.

5.1.3 Carbon Source

Plants in tissue culture grow heterotrophically and since they cannot synthesize sufficient of their own carbon source, one (usually sucrose) must be included in the medium. The carbon source provides energy for plant growth as well as providing the building blocks for the production of larger molecules needed for plant growth.

Usually sucrose at a concentration of 1-5% is used as the source of carbon but other carbon sources which have been used include glucose, maltose, galactose and lactose. When sucrose is autoclaved there is a certain amount of hydrolysis to produce some glucose and fructose which may be used more efficiently by plants in culture.

5.1.4 Agar

Some plants or their tissues are cultured in liquid medium which may be static or usually agitated on a shaker. Most tissues are cultured on solid medium which is gelled using agar or an agar substitute, e.g. Gelrite or Phytagel. The agar concentration used is in the range 0.7 -1.0%. At high concentrations the agar becomes very hard, there is little available water and as a result there is poor diffusion of nutrients into the plant tissues. High quality agar such as Difco BiTek agar is expensive but contains fewer impurities which may inhibit plant growth. Other substitutes such as gelatine are sometimes used in commercial laboratories.

Synthetic gels are known to cause vitrification which is a physiological problem occurring during tissue culture of many species. To overcome this problem a new product called Agargel has been produced by Sigma. This product is a mixture of agar and the synthetic gel and offers the best attributes of both products while reducing the problems of vitrification. This product can be made in the laboratory by mixing 1 g of Gelrite (Phytagel) with 4 g of agar in 1 L of water.

5.1.5 pH

The pH of the medium is normally adjusted in the range of 5.6 to 5.8 but different plants may require a different pH for optimum growth. If the pH is higher than 6.0 the medium may become too hard and if the pH is less than 5.2 problems are usually encountered with gelling.

5.1.6 Growth Regulators

In most media one or more growth regulators are added. The growth regulators are discussed in detail in the Chapter 6.

5.1.7 Water

Normally glass distilled water is used in tissue culture, with many laboratories using double distilled water. The use of ion exchange columns has been criticized (Dodds and Roberts 1985) because of the release of a variety of organic contaminants during the process. However ion exchange columns provide the water for many laboratories without problems and are used widely in Europe. Some laboratories, in the interests of economy, use untreated rainwater but this provides less control over the inorganic and organic content of the medium.

5.2 Selection of Media

Since most plant species require different media, the selection of the most suitable one poses particular problems. The best place to start is to consider published methods: the texts by de Fossard (1976) and George and Sherrington (1984) are excellent starting points.

If no information is available it is usual to start with the MS medium (Murashige and Skoog 1962). While this medium has a higher concentration of salts relative to most other media and is also high in nitrate, it has been used successfully with a wide variety of dicotyledonous plants. For callus initiation 2,4-D is added to the medium in concentrations ranging from 1 to 5 $mg.L^{-1}$. For shoot multiplication, a cytokinin such as BAP is added often with the addition of low concentrations of an auxin such as NAA. For root initiation, IBA at concentrations of 1-2 $mg.L^{-1}$ is added. The factors which are most difficult to determine in tissue culture media are the growth regulators and it may be

necessary to devise an experiment to determine the best concentration to use. There are two approaches:

One approach is to accept that the MS medium is appropriate and investigate a range of say four concentrations of two different growth regulators. See Table 5.1.

Table 5.1 An experimental approach used to select the most useful concentrations of the growth regulators BAP and NAA as additives to an MS medium containing 2% sucrose and 0.8% agar. Modified from Bhojwani and Razdan (1983).

		BAP Concentration mg.L^{-1}			
		0	0.5	2.5	5.0
NAA	0	1	2	3	4
Concentration	0.5	5	6	7	8
mg.L^{-1}	2.5	9	10	11	12
	5.0	13	14	15	16

The second approach is to use the broad spectrum method of de Fossard (1976) where four different categories, Minerals, Auxins, Organics and Cytokinins, are examined at each of three concentrations. This very large experiment requires 81 different treatments and is very time consuming but may be a necessary approach with some plants that prove to be very difficult to culture (discussed later).

One can follow a couple of handy hints to increase the probability of success in getting explants started. First, if no formula can be found for culturing the plant in hand, or the prescribed formula is ineffective, try various standard media (both liquid and solid) starting with those that have been used successfully for related plants (the same family or genus). Another useful approach is to start herbaceous perennials in half strength MS salts without hormones while woody plants could be tried in McCown's Woody Plant Medium (WPM) without hormones.

5.3 Media Preparation

The most widely used medium in plant tissue culture is that of Murashige and Skoog (1962). The most convenient way to prepare MS medium is to purchase commercially available prepackaged media from companies such as the Sigma Chemical Company. The following steps are used to make 1 L of MS medium from either commercially prepared media (a) or from stock solutions (b).

(a) Preparation of MS medium from commercial packages

i. Dissolve the contents of a packet for the preparation of 1 L of medium with stirring into 600 mL of distilled water: rinse out the packet with distilled water.

ii. Add cytokinins and/or auxins if required. (It is convenient to make up stock solutions containing 100 mg of the growth regulator in 100 mL of distilled water. This can then be stored in the refrigerator.) One mL of growth regulator stock solution added to 1 L of medium will give a concentration of 1 mg L^{-1} of that growth regulator.

iii. Weigh out and add any other supplements.

iv. Add sucrose, usually 30 g L^{-1}.

v. Make up volume to 1 litre.

vi. Adjust pH to 5.8, using KOH or HCl.

vii. Add agar (or other gelling agent) usually 6-10 g L^{-1} and heat with stirring until thoroughly dissolved.

viii.
- Dispense into suitable culture vessels, autoclave and cool,

  OR
- Autoclave in bulk, cool to about 50°C then dispense into sterile culture vessels or containers.

(b) Preparation of MS medium from Stock Solutions prepared from laboratory chemicals.

The preparation of MS (and other) media from basic chemicals is time consuming and less convenient but it does have the advantage of providing the flexibility to vary individual components of the medium. It may also be cheaper than using proprietary mixes.

(i) Preparation of Stock Solutions

The preparation of MS media by this method is based on four concentrated stock solutions which are prepared and held in the refrigerator.

- Stock Solution 1 Macronutrients (10 x concentration)
  (sufficient to make 10 L of MS media)

  The following chemicals are dissolved in one L of distilled water:

NH_4NO_3	16.5 g
KNO_3	19.0 g
$CaCl_2,2H_2O$	4.4 g
$MgSO_4,7H_2O$	3.7 g
KH_2PO_4	1.7 g

- Stock Solution 2 Micronutrients (100 x concentration)
  (sufficient for 100 L)

  Add the following chemicals to one L of distilled water:

$MnSO_4,4H_2O$	2230 mg
$ZnSO_4,4H_2O$	860 mg
H_3BO_3	620 mg
KI	83 mg
$Na_2MoO_4,2H_2O$	25 mg
$CuSO_4,5H_2O$	2.5 mg
$CoCl_2,6H_2O$	2.5 mg

- Stock Solution 3 Vitamins (100 x concentration)
(sufficient for 10 L)

  Add the following vitamins to 100 mL of distilled water.

Glycine	20 mg
Nicotinic acid	5 mg
Pyridoxine HCl	5 mg
Thiamine HCl	1 mg
Myo-Inositol	1000 mg

- Stock Solution 4 Fe-EDTA (100 x concentration)
(sufficient for 10 L)

  Add the following to 100 mL of distilled water:
  $FeSO_4,7H_2O$ 278 mg and
  $Na_2EDTA,2H_2O$ 373 mg

  Dissolve EDTA first then Iron.
  Store in a dark bottle at 0-4°C.

(ii) **Preparation of 1 L of MS medium from stock solutions.**

1. To 600 mL of distilled water, with stirring:

   (a) add 100 mL of macronutrient Stock Solution 1.
   (b) add 10 mL of micronutrient Stock Solution 2.
   (c) add 10 mL of vitamin Stock Solution 3.
   (d) add 10 mL of Fe-EDTA Stock Solution 4.

2. Add cytokinin and/or auxins as necessary.

3. Add sucrose (usually 30 $g.L^{-1}$).

4. Make up volume to 1 L with distilled water.

5. Adjust pH to 5.8.

6. Add agar, (usually 6-10 $g.L^{-1}$) - dissolve.

7. Dispense into suitable culture vessels and autoclave.

(c) MEDIA PREPARATION - DE FOSSARD'S METHOD

Four broad categories of constituents are tested in deFossard's "Broad spectrum method".

1. Minerals - same elements as M & S

2. Auxins - Combination of 6 auxins are used - IAA, IBA, NAA+
   3 oxygenated auxins p CPA (para - chlorophenoxy acetic acid)
   NOA (2, Naphthoxy acetic acid)
   and 2,4-D

3. Cytokinins: BAP and kinetin

4. Organics (growth factors + amino acids + sucrose):

Apart from those already included in M & S medium following are also incorporated in de Fossard's media.

- Biotin
- D-ca-pantothenate
- Riboflavin
- Abscorbic acid
- Choline chloride
- Glycine
- L-Cystein HCl

Four levels of each constituent are used:

Z = zero
L = low
M = medium
H = high

Minerals	L	$\xrightarrow{X5}$	M	$\xrightarrow{X5}$	H
Auxins	L	$\xrightarrow{X10}$	M	$\xrightarrow{X10}$	H
Cytokinins	L	$\xrightarrow{X10}$	M	$\xrightarrow{X10}$	H
Organics	L	$\xrightarrow{X5}$	M	$\xrightarrow{X2}$	H
Sucrose	L	$\xrightarrow{X10}$	M	$\xrightarrow{X2}$	H

There are 12 Group Stock Solutions (G.S.S.) as per Table 5.2.

TABLE 5.2 Broad spectrum stock solution concentrations and codes

Group Stock Solutions	Concentration L	M	H
Minerals	#1	#2	#3
Auxins	#4	#5	#6
Cytokinins	#7	#8	#9
Organics	#10	#11	#12

(d) ALTERNATIVE METHOD

We have simplified de Fossard's method of media preparation to that of the following 3 groups:

Group I	-	Macronutrients + Iron Stock Solution (10x Concentration)
Group II	-	Micronutrient Stock Solutions (100x Concentration)
Group III	-	Organics (100x Concentration)

These groups can be prepared as follows:

(i) GROUP I

Macronutrients + Iron Stock Solution (10x Concentration)

Dissolve the following chemicals in 700 mL of distilled water, then add water to final volume of 1 L.

NH_4NO_3	16.01 g
$K\ NO_3$	20.222 g
$MgSO_4,7H_2O$	7.395 g
$Na\ H_2\ PO_4,2H_2O$	3.1202 g
$Ca\ Cl_2,2H_2O$	4.4106 g
Fe Na EDTA	0.36707 g

To prepare media with H, M or L levels add 100, 50 or 10 mL respectively to one litre of distilled water.

(ii) GROUP II

Micronutrient (100x Concentration)

Dissolve the following chemicals first in 400 mL of distilled water then add water to final volume of 500 mL.

$H_3\ BO_3$	0.4638 g
M_nSO_4,H_2O	0.8451 g
$Z_nSO_4,7H_2O$	0.5751 g
KI	0.0415 g
$C_u\ SO_4,5H_2O$	0.00125 g
$Na_2\ M_oO_4,2H_2O$	0.0125 g
$CoCl_2,6H_2O$	0.00125 g

To prepare media with H, M or L levels add 10, 5 or 1 mL respectively to one litre of H_2O.

(iii) GROUP III

Organics (100x Concentration)

Dissolve the following chemicals in 400 mL of distilled water. Heat is required to dissolve Riboflavin. When cool make up to the final volume of 500 mL.

Inositol	5.405 g
L. Cystein HCl	0.946 g
Nicotinic Acid	0.246 g
Pyridoxine, HCl	0.062 g
Thiamine, HCl	0.6745 g
Biotin	0.0122 g
D-Ca-Pantothenate	0.1192 g
Riboflavin	0.1882 g
Ascorbic Acid	0.088 g
Choline chloride	0.0697 g
Glycine	0.1877 g

To prepare media with H, M or L levels add 10, 5 or 1 mL respectively to one litre of H_2O.

(iv) Example: Preparation of 1 L of MZZM.

1. To 500 mL of distilled water, with stirring:-

   (a) add 50 mL of Group I
   (b) add 5 mL of Group II
   (c) add 5 mL of Group III

2. Add Sucrose* 20.539 g which is equivalent to 60 mM.

3. Make up to one litre with distilled water.

4. Adjust pH to 5.5

5. Add agar, BiTeck, 8 gL^{-1}, dissolve.

6. Dispense into suitable vessels and autoclave.

(e) Additional notes:

1. de Fossard expresses media ingredients in moles. The advantage of this unit is that moelcules and ions interact as entities, thus, comparing moles instead of weight provides a more valid basis of comparison of one formula with another.

2. Inorganic stocks are more stable than organic stocks. As a general rule store all stocks in a fridge. Inorganics could be stored at room temperature but run the risk of growing contaminants because of the warm temperature. If the salt solutions are bordering on forming precipitates they will certainly do so in the cold because the solubility of most compounds decreases with the temperature. Should they form precipitates they can be brought up to room temperature or gently warmed. Do not heat excessively because evaporation will alter the concentration of salt. As a general rule store auxins and cytokinins for 1 month, all other stocks no longer than 3 months. If sediment or contaminant appears the solution should be discarded immediately.

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*de Fossard's

Low Sucrose	=	2.054 g/L	=	6 mM.
Medium Sucrose	=	20.539 g/L	=	60 mM.
High Sucrose	=	40.077 g/L	=	120 mM.

3. Stock solutions should be prepared in such a way as to not form precipitates. None of the precipitated chemicals can be used by the plants. In order to avoid the formation of precipitates when making stock solutions one of two alternative strategies can be followed.

   a. Combine only those compounds which do not form precipitates at high concentrations.

   b. Make the stock solutions weak enough that precipitates will not form.

4. Points of interest.

- **Iodine:** an element not essential to growth of higher plants, is likely to be involved in auxin transport.
- **Manganese:** has been found to have a role as a cofactor in oxidising IAA.
- **Zinc:** has been suggested to prevent oxidation of auxin. It seems to be necessary for synthesis of tryptophan, a precursor of auxin that affects auxin levels.
- **Inositol:** One of vitamin B complex, described as a "sugar alcohol" in its phosphate form. It is part of various membranes particularly those of chloroplasts.
- **Thiamine:** Vitamin B_1 - Functions as a coenzyme to assist the organic acid cycle of respiration (Krebs cycle).
- **Nicotinic acid:** (Niacin) is a constituent of coenzymes active in light energy reactions.
- **Pyridoxine:** Vitamin B_6 also serves as a coenzyme in metabolic pathways (chemical reactions of metabolism).
- **Pantothenic acid:** Another B Vitamin, is active as a coenzyme in fat metabolism.
- **Choline:** Is an alkaloid within Vitamin B complex.
- **Riboflavin:** Vitamin B_2 - Functions as coenzyme - a blue light receptor pigment has the ability to desensitize the photoxidation of the auxin IAA.
- **Biotin:** (Vitamin H) is active as a coenzyme in fat metabolism.
- **L-Cystein**
- **Glycine**

  Both these amino acids are used in de Fossard's media. Amino acids are building blocks of proteins and nucleic acids.

- **Agar:** Concentration of impurities varies with the source of algae and method of manufacture.

  e.g. chloride content in Spanish sources is 3.1% whereas only a trace in Japanese and Portuguese ones.

- **Ascorbic acid:** An antioxidant agent used in retardation of tissue browning.

Table 5.3 A summary of the functions of inorganic nutrients in plants from Raven *et al.* 1986.

ELEMENT	PRINCIPAL FORM IN WHICH ELEMENT IS ABSORBED	USUAL CONCENTRATION IN HEALTHY PLANTS (% OF DRY WEIGHT)	IMPORTANT FUNCTIONS
Macronutrients			
Carbon	CO_2	~44%	Components of organic compounds.
Oxygen	H_2O or O_2	~44%	Component of organic compounds.
Hydrogen	H_2O	~6%	Component of organic compounds.
Nitrogen	NO_3 or NH_4+	1-4%	Amino acids, proteins, nucleotides, nucleic acids, chlorophyll, and coenzymes
Potassium	K^+	0.5-6%	Enzymes, amino acids, and protein synthesis. Activator of many enzymes. Opening and closing of stomata.
Calcium	Ca^{2+}	0.2-3.5%	Calcium of cell walls. Enzyme cofactor. Cell permeability. Component of calmodulin, a regulator of membrane and enzyme activities.
Phosphorus	$H_2PO_4^-$ or HPO_4^{2-}	0.1-0.8%	Formation of "high-energy" phosphate compounds (ATP and ADP). Nucleic acids. Phosphorylation of sugars. Several essential coenzymes. Phospholipids.
Magnesium	Mg^{2+}	0.1-0.8%	Part of the chlorophyll molecule. Activator of many enzymes.
Sulfur	SO_4^{2-}	0.05-1%	Some amino acids and proteins. Coenzyme A.

Table 5.3 Continued

ELEMENT	PRINCIPAL FORM IN WHICH ELEMENT IS ABSORBED	USUAL CONCENTRATION IN HEALTHY PLANTS (% OF DRY WEIGHT)	IMPORTANT FUNCTIONS
Micronutrients			
Iron	Fe^{2+} or Fe^{3+}	25-300 ppm	Chlorophyll synthesis, cytochromes, and nitrogenase.
Chlorine	Cl^-	100-10,000 ppm	Osmosis and ionic balance; probably essential in photosynthetic reactions that produce oxygen.
Copper	Cu^{2+}	4-30 ppm	Activator of certain enzymes.
Manganese	Mn^{2+}	15-800 ppm	Activator of certain enzymes.
Zinc	Zn^{2+}	15-100 ppm	Activator of many enzymes.
Molybdenum	MoO_4^{2-}	0.1-5.0 ppm	Nitrogen fixation. Nitrate reduction.
Boron	BO_3^- or $B_4O_7^{2-}$	5-75 ppm	Influences Ca^{2+} utilization. Functions unknown.
Elements Essential to Some Plants or Organisms			
Cobalt	Co^{2+}	Trace	Required by nitrogen-fixing micro organisms
Sodium	Na^+	Trace	Osmotic and ionic balance, probably not essential for many plants. Required by some desert and salt-marsh species. May be required by all plants that utilize C_4 photosynthesis.

CHAPTER 6

PLANT GROWTH REGULATORS IN TISSUE CULTURE

Hormones are organic compounds which are synthesized in the tissues of plants. They are required in only very low concentrations to influence plant growth and development. Many synthetic organic molecules have been found which have biological activity similar to the hormones. As a group the synthetic compounds and the naturally occurring hormones are known as growth regulators.

Tissue culture is the manipulation of plant growth under carefully controlled conditions and the auxins and cytokinins are of particular significance. Most explants produce some (endogenous) auxin and cytokinin. In tissue culture additional (exogenous) growth regulators are applied to achieve the growth effect. As a general guide either auxin or cytokinin or both auxin and cytokinin are added to the culture to achieve the desired growth response.

Some practical aspects of the use of growth regulators.

Growth regulators for use in media are stored in the dark in the refrigerator as stock solutions. Small volumes (say 50 mL) of concentrated solutions containing 1 mg mL^{-1} of the growth regulator may be stored for some time in this manner. The stability of growth regulators varies; kinetin and IAA are unstable in the light so are usually stored in dark bottles. Also, IAA loses its activity in aqueous solutions so stock solutions of IAA should not be kept for long periods.

De Fossard (1976) provides very useful details on preparation of stock solutions. In general auxins should be dissolved in a small volume of 95% alcohol before the correct volume is obtained by the addition of water. Cytokinins are dissolved in a small volume of 1 N hydrochloric acid and then water is added to achieve the final volume. The Sigma Plant Tissue Culture Catalogue provides considerable detail on media preparation. This catalogue recommends that all the cytokinins and auxins used in micropropagation be first dissolved in 1N sodium hydroxide. Appendix 8 provides detailed information on a wide range of plant growth regulators in use in plant tissue culture.

6.1 Auxins

This group includes the naturally occurring IAA and the synthetic auxins IBA, NAA and 2, 4-D (see Figure 6.1 for the chemical structures of these compounds). The auxins are added to the nutrient medium in concentrations within the range of 0.01-10 mg L^{-1}. The synthetic auxins are relatively more active than IAA and have the added advantage that they are not degraded by enzymes present in the plant tissues.

Auxins have a number of different roles in plant growth and development. They stimulate cell elongation and growth, cell division particularly in callus formation and the formation of adventitious roots. Auxins also inhibit axillary bud development and the formation of somatic embryos from callus cultures.

6.2 Cytokinins

These include the naturally occurring 2iP and zeatin and the synthetic cytokinins BAP and kinetin (see Figure 6.1). The synthetic cytokinins have very high biological activity and are not expensive so have wide application in tissue culture. Cytokinins cause tissues to swell, induce the development of axillary buds and the adventitious buds and promote cell

division. Their role in inducing axillary bud development through decreasing apical dominance is very important in tissue culture. Cytokinins are heat stable and may be added to the medium prior to autoclaving.

6.3 Gibberellic Acid

This group includes about sixty compounds of which GA_3 (see Figure 6.1) is most widespread in plants. Gibberellic acid is not frequently used in tissue culture work. It is heat labile and cannot be autoclaved and thus must be added to the medium after autoclaving using millipore filtration (filter sterilization). The general role of gibberellic acid is to promote seed germination and to induce elongation of the internode. It has been used in culture media to facilitate elongation of very small shoots and to stimulate embryo formation from callus (Figure 6.1).

6.4 Abscisic Acid

Abscisic acid (ABA see Figure 6.1) is found widely distributed in plant tissues and is regarded as being a growth inhibitor. It is not often used in tissue culture but it has specific applications such as stimulating embryoid development from callus (George and Sherrington 1984). These roles for ABA may be related to its ability to modify cytokinin synthesis and as a gibberellin antagonist.

6.5 Ethylene

Ethylene is a gas of simple structure (Figure 6.1). It is produced by plants and in closed culture vessels the accumulation of ethylene is believed to result in growth inhibition (George and Sherrington 1984). Ethylene is also produced by some plastic containers and flaming also produces some ethylene. It appears to interact with other growth regulators and is implicated in the senescence of plant tissues. Ethylene is produced in response to waterlogging, a condition analogous to *in vitro* culture. At higher concentrations ethylene is known to induce vitrification.

6.6 Other Substances

6.6.1 Adenine

Adenine is frequently added to culture media and has been shown to have a beneficial effect on shoot production. While the mode of action of adenine is unknown it is likely that it exerts its effects through allowing greater synthesis of natural cytokinins for which it is a precursor.

6.6.2 Activated carbon

Is used at concentrations from 0.2 to 3% in culture media. Activated carbon has the capacity to remove substances from the medium by adsorption, thus making these substances unavailable to the tissues. It has been shown that activated carbon will adsorb growth regulators from the medium (George and Sherrington 1984) as well as other substances such as polyphenols which are toxic to cell growth. This suggests that the growth regulator role proposed for activated carbon is related to its absorptive properties rather than any direct effect. Activated carbon is often used in root induction media where its role may be related to reducing light to enhance root growth as well as absorption of growth regulators (see Appendix 6 for more details).

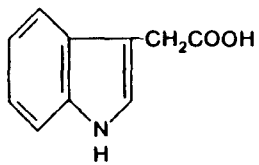
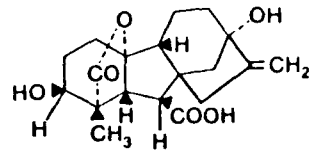
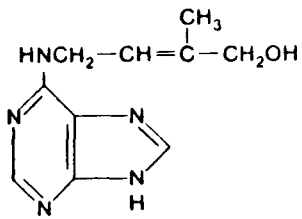
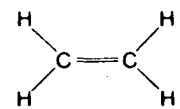
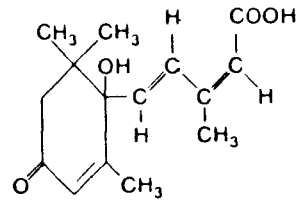
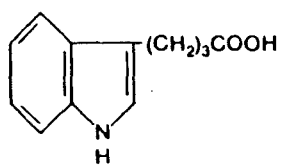
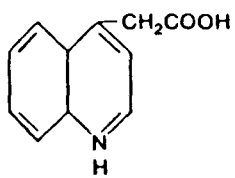
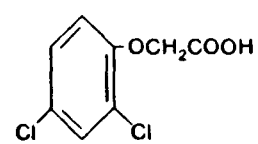
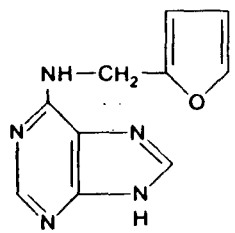
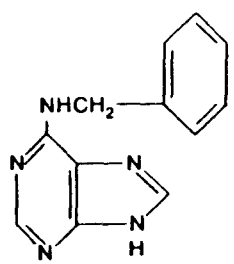
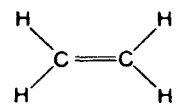
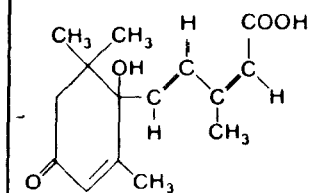
	AUXIN	GIBBERELLIN*	CYTOKININ	ETHYLENE	ABSCISIC ACID
NATURALLY-OCCURRING	 Indoleacetic Acid (IAA)	 GA_3 (Gibberellic Acid)	 Zeatin	 Ethylene	 Abscisic Acid
SYNTHETIC	 Indolebutyric Acid (IBA)  a-Naphthaleneacetic Acid (NAA)  2,4-Dichlorophenoxyacetic Acid (2,4-D)	*There are 52 or more forms of gibberellin, all of which slightly differ only in structure from the GA_3 shown here.	 6-Furfurylamino Purine (kinetin)  6-Benzylamino Purine (BA)	 Ethylene	 Abscisic Acid

Fig. 6.1 Commonly used plant growth regulators in tissue culture.

CHAPTER 7

COMMERCIAL ASPECTS OF MICROPROPAGATION

7.1 Introduction

Micropropagation as a technique has considerable commercial appeal because one can produce large numbers of plants of new varieties quickly or to produce disease free stock. The techniques are now well established and there is a large tissue culture literature providing detailed information on the culture of individual plant species. There are now a large number of businesses in Australia using well established protocols to propagate a variety of plant species. Most of these are quite small operations and produce only small profits. The potential for commercial tissue culture will not be realized unless profit margins improve. This section discusses some of the reasons for low profits and failure of commercial tissue culture operations.

7.2 The reasons for low profitability are many but include:

(a) lack of knowledge
(b) inadequate facilities
(c) poor management and business skills
(d) poor product
(e) lack of marketing skills
(f) high costs of commercial micropropagation

7.2.1 Lack of Knowledge

Many operators enter commercial micropropagation with inadequate knowledge of tissue culture. To be successful the operator needs to have a basic understanding of the factors affecting plant growth, how plants may respond in culture and how their growth can be manipulated. A sound basis of plant function will allow the operator to modify procedures to maintain high rates of plant production. There are a number of comprehensive tissue culture courses offered in Australia which provide sufficient background and participants should expect to pay $400 to $700 for such courses. The course that is offered for one afternoon for $50 perhaps provides enough information to satisfy the casual curiosity of hobbyists but is a poor basis upon which to establish a business.

7.2.2 Poor Facilities

While micropropagation for the hobbyist can be carried out on the kitchen table and using a domestic pressure cooker to sterilize media and materials, such an approach is inadequate for a commercial operation. Appendix 1 lists the items which are required for a well equipped laboratory used for micropropagation. Depending upon the area available to site the laboratory, the cost of establishment would probably exceed $50,000. However, considerable savings can be made with the purchase of reliable second hand items.

7.2.3 Inadequate Finance and Poor Management and Business Skills

Although the techniques of micropropagation are comparatively simple, success depends upon having adequate reliable facilities which are capable of producing the large numbers of plants necessary to meet advance orders. Sufficient finance is necessary to provide and maintain adequate facilities for the operation. Management of the facilities and staff needs to be geared to the desired level of production.

7.2.4 Quality of Product

To succeed in the competitive market place, plants need to be of high quality and true to type. Quality can be achieved by attention to detail such as cutting procedure, frequency of transfer, suitability of media and control of the environment in the plant growth area. Too many laboratories are unaware of the potential dangers of mutation that can occur in tissue culture. While this is not such a problem with ornamental foliage plants which are sold after the foliage type has developed, it is a very serious problem with other plants such as bananas and oil palm in which the non productive mutants may not be detected in culture. Such plants will only be found after some time in the field. The result is a financial loss to the commercial grower and potential litigation case against the tissue culture laboratory. Irresponsible operators may provide an unreliable product but a more serious aspect of their activities is the fact that large industries such as the banana industry lose confidence in tissue cultured plants. It is industries such as the commercial banana industry that have so much to profit from tissue culture through access to disease free plants from high producing parents.

7.2.5 Lack of Marketing Skills

A tissue culture laboratory established to supply certain types of plants specifically to one nursery encounters few marketing problems. A laboratory established to produce general or specific lines of plants for sale to the general nursery trade encounters an already very competitive market. At this level of business it is the very efficient laboratories, which are able to produce quality plants efficiently to keep their costs per unit low and have marketing expertise, that survive.

7.2.6 Production Costs

A serious limitation to the extension of commercial tissue culture techniques to new crops such as forestry species and vegetables is the high cost of production per propagule. In 1977, Anderson *et al.* produced figures for the cost of producing broccoli to a stage ready for transplanting into the field using micropropagation. These data are from the United States and are based on the production of 11,000 plants per week. These data are shown in Table 7.1 and divided the costs into two parts. Of the micropropagation component, wages and salaries account for 8.8 cents per explant or 57% of the total cost.

Although these data were published in 1977 they still provide very useful information. Media costs still approximate to 0.6 c per plant, while culture vessels may cost as much as 2 c per plant if commercially available bottles are used. With increased wage costs the costs per plant are now probably close to 18 c.

A very comprehensive coverage of the economics of commercial tissue culture is provided by de Fossard (1990). It is specifically written for the Australian trade so may be considered mandatory reading for anyone seriously considering entering the plant tissue culture business in this country.

Levin and Vasil (1989) have considered the ways in which costs of micropropagation may be reduced in the future, under the following topics.

(a) Cost reduction in the multiplication phase.
(b) Cost reduction in the growth stage.
(c) Cost reduction in the greenhouse stage.
(d) Cost reduction in transfer of propagules to the field.

Table 7.1 Costs of producing broccoli to transplanting stage using micropropagation techniques. Figures are based on a production of 11,000 plants per week. (Taken from Anderson *et al.* 1977).

			Cost per unit	% of total
1.	Tissue Culture Production			
	A	Wage and salary	8.8 c	57.1
	B	Media	0.6 c	3.8
	C	Culture vessels	0.2 c	1.3
	D	Equipment and laboratory	1.4 c	9.1
	E	Overheads	0.6 c	3.8
	TOTAL TISSUE CULTURE COSTS		11.6 c	75.3
2.	Greenhouse Production			
	A	Wages	1.3 c	8.3
	B	Flats and soil mix	0.3 c	1.9
	C	Bench charge	2.2 c	14.3
			3.8 c	24.5
	TOTAL COST PER PLANT =		15.4 c	100.0

These cost reductions will be achieved through more efficient manipulation of the plant in culture as well as the use of labour saving devices. They conclude that it is likely that the automation of the multiplication phase of tissue culture will be feasible within the next few years for a number of species of plants including potato microtubers, lily bulblets, some ornamental plants and a few vegetables. The commercial success of automated, propagation will result in the rapid adoption of the technique to suitable species where hundreds of millions of propagules may be required each year.

7.3 The Future for Commercial Micropropagation with Current Technology

The comments offered above perhaps are discouraging to newcomers to the industry. There is certainly a need to recognize the many potential pitfalls that await the unprepared person entering commercial tissue culture. Despite these difficulties there are a number of commercial tissue culture laboratories that are successful businesses. Undoubtedly some newcomers to the industry will also be highly successful. Where then do the opportunities lie? An opportunity exists for the nurseryman who already has a market for his plants to establish a small laboratory to service his own needs in a few plant species. One can see opportunities for specialist tissue culture laboratories which produce one or a very few species for specialized industries such as the cut flower trade. There are undoubtedly opportunities to market certain types of plants on the American and particularly European markets but these however are fraught with marketing problems. As well the high cost of labour input which is common to so many Australian products and which makes competition in the international market place so difficult.

APPENDICES

APPENDIX 1

APPARATUS AND EQUIPMENT

The following is a list of apparatus and equipment required for tissue culture work. The items listed would be suitable for equipping a small commercial laboratory.

5.4.1 Glassware for media preparation:

- Flasks (100 mL, 250 mL, 500 mL, 1 L, 5 L).
- Volumetric flasks (500 mL, 1 L, 2 L, 3 L).
- Measuring cylinders (25 mL, 50 mL, 100 mL, 1 L).
- Graduated pipettes (1 mL, 2 mL, 5 mL, 10 mL).
- Pasteur pipettes.
- Culture vials (culture tubes, screw-cap bottles of various sizes, petri-dishes, etc.) with suitable closure.

5.4.2 Other laboratory items:

- Drying oven.
- Wire-mesh baskets, to autoclave media in small vials and for drying labware.
- Water distillation unit or demineralization unit, to obtain high quality water.
- Plastic containers (10 L and 20 L), to store high quality water.
- Balance, to weigh small quantities.
- Balance, to weigh larger quantities.
- Hot plate - magnetic stirrer, to dissolve chemicals.
- Plastic bottles of different sizes, to store and deep-freeze solutions.
- pH meter, to adjust pH of media and solutions.

5.4.3 Major equipment items:

- Laminar flow cabinet.
- Air conditioner to maintain culture room temperature.
- Binocular stereoscopic microscope for examination of cultures.
- Refrigerator and freezer for storage of stock solutions and chemicals.
- Autoclave for sterilization.
- Growing racks fitted with fluorescent lights for the culture room.

5.4.4 Small items for use mainly in the clean area:

- Filter membranes to filter sterilize solutions.
- Hypodermic syringes, for filter sterilization of solutions.
- Trolley with suitable trays, to transport cultures, media and apparatus.
- Spirit lamp or bunsen burner, to flame instruments.
- Atomizer, to spray alcohol in the inoculation chamber.
- Instrument stand to support sterilized instruments during aseptic manipulations.
- Large forceps with blunt ends, for inoculation and subculturing.
- Fine needles, for dissections.
- Scalpels for cutting plant material.
- Scissors.

APPENDIX 2

COMMONLY USED MEDIA

All figures are $mg.l^{-1}$

Ingredients	MS[1]	B5[5]	WMP[3]	WH[4]	SH[5]
$(NH_4)NO_3$	1,650		400		
$(NH_4)_2SO_4$		134			
$(NH_4)H_2PO_4$					300
KNO_3	1,900	2,500		80	2,500
$Ca(NO_3)_2.4H_2O$			556	208.4	
$CaCl_2.2H_2O$	440	150	96		200
$MgSO_4.7H_2O$	370	250	370	360	400
Na_2SO_4				200	
KH_2PO_4	170		170		
$NaH_2PO_4.H_2O$		130.5		16.5	
KCl				65	
K_2SO_4			990		
$FeSO_4.7H_2O$	27.8	27.8	27.8	2.5	15
Na_2EDTA	37.26	37.3	33.6		20
$MnSO_4.4H_2O$	22.3				
$MnSO_4.H_2O$		10	22.3	3.8	10
$ZnSO_4.7H_2O$	8.6	2.0	8.6	3.0	1.0
H_3BO_3	6.2	3.0	6.2		5.0
KI	0.83	0.75		0.75	1.0
$NaMoO_3$				0.001	
$Na_2MoO_4.2H_2O$	0.25	0.25	0.25		0.1
$CuSO_4.5H_2O$	0.025	0.025	0.25	0.001	0.2
$CoCl_2.6H_2O$	0.025	0.025			0.1
myo-inositol	100	100	100		1,000
nicotinic acid	0.5	1.0	0.5	0.5	5.0
pyridoxine.HCl	0.5	1.0	0.5	0.1	0.5
thiamine.HCl	0.1	10.0	1.0	0.1	5.0
glycine	2.0		2.0	3.0	
sucrose	30,000.0	20,000.0	20,000.0	20,000.0	30,000.0

[1]Murashige, T. and F. Skoog (1962).
[2]Gamborg, O.L., Millers, R.A. and K. Ojima (1968).
[3]McCowan, B. and G.B. Lloyd (1982).
[4]White, P.R. (1954).
[5]Schenk, R.V. and Hildebrandt, A.C. (1972).

APPENDIX 3

PREPARATION OF CONCENTRATED CHEMICAL STOCK SOLUTIONS
(From de Fossard 1976)

The units of measurement in tissue culture media preparation are: litres, millilitres, microlitres, milligrams, micrograms, moles, millimoles, micromoles, and molar, millimolar and micromolar.

Table 1

UNITS OF MEASUREMENT

1 L (= LITRE)	=	1,000 ml (= MILLI-LITRES)
	=	1,000,000 µl (= MICRO-LITRES)
1 g (= GRAM)	=	1,000 mg (= MILLI-GRAMS)
	=	1,000,000 µg (= MICRO-GRAMS)
1 mole or mol (= GRAM-MOLECULAR WEIGHT)	=	1,000 mmol (= MILLI-MOLES)
	=	1,000,000 µmol (= MICRO-MOLES)
1 M (= MOLAR)	=	1 mol in 1 L
	=	1,000 mmol in 1 L
	=	1,000,000 µmol in 1 L
1 mM (= MILLI-MOLAR)	=	1 mmol in 1 L
	=	1,000 µmol in 1 L
1 µ (= MICRO-MOLAR)	=	1 µmol in 1 L

Table 2

PARTS PER MILLION AND PERCENTAGES

p.p.m. (= **PARTS PER MILLION**) = PARTS (ANY UNIT) OF A SUBSTANCE IN 1,000,000 PARTS (SAME UNIT) OF ANOTHER SUBSTANCE.

Examples (1) 5,000 ppm IBA (in talc)
= 5,000 mg IBA + 995,000 mg talc = 1,000,000 mg

(2) 10 ppm IBA (in medium)
= 10 mg IBA + 990,000 mg H_2O (= 990 mL H_2O)

IN PRACTICE, 10 mg IBA placed in flask and H_2O is added to 1 litre mark (= 10 ppm IBA).

% (= **PERCENTAGE**)

(1) % (V/V) = PARTS (in mL) of one substance in 100 PARTS (mL) OF ANOTHER SUBSTANCE.

Example: 0.1% (v/v) Tween Twenty
= 1 mL Tween Twenty $\xrightarrow{+H_2O}$ 1,000 mL

(2) % (w/v) = PARTS (in g) OF ONE SUBSTANCE IN 100 PARTS (mL) OF ANOTHER SUBSTANCE

Example: 0.001% (w/v) IBA (= 10 ppm IBA)
= 0.01 g IBA $\xrightarrow{+H_2O}$ 1,000 mL

Table 2 describes parts per million and percentage as used in some tissue culture practices.

Table 3

MOLE

Sucrose is used as an example to define a mole, millimole, and micromole.

MOLE (mol) = GRAM-MOLECULAR WEIGHT

EXAMPLE

SUCROSE, $C_{12}H_{22}O_{11}$

1 mol $C_{12}H_{22}O_{11}$	=	(12 x 12.011) + (22 x 1.008) + (11 x 16.000)
	=	(144.132) + (22.176) + (176.000)
	=	342.308 g
$\therefore$ 1 mol $C_{12}H_{22}O_{11}$	=	342.31 g $C_{12}H_{22}O_{11}$
1 mmol $C_{12}H_{22}O_{11}$	=	342.31 mg $C_{12}H_{22}O_{11}$
1 µmol $C_{12}H_{22}O_{11}$	=	342.31 µg $C_{12}H_{22}O_{11}$
1 mol $C_{12}H_{22}O_{11}$	=	6.023×10^{23} MOLECULES $C_{12}H_{22}O_{11}$
1 mmol $C_{12}H_{22}O_{11}$	=	6.023×10^{20} MOLECULES $C_{12}H_{22}O_{11}$
1 µmol $C_{12}H_{22}O_{11}$	=	6.023×10^{17} MOLECULES $C_{12}H_{22}O_{11}$

Table 4

Ions are partly described in this table by means of an example when three compounds are mixed together.

IONS

EXAMPLE

100 mL 10 mM NH_4NO_3
\+ 100 mL 10 mM KNO_3
\+ 100 mL 10 mM $Ca(NO_3)_2$
= 300 mL SOLUTION OF:

1 MMOL NH_4^+
1 mmol K^+
1 mmol Ca^{2+}
4 mmol NO_3^-

EXPLANATION

(1) 1000 mL 10 mM NH_4NO_3 CONTAINS 10 mmol NH_4NO_3
$\therefore$ 100 mL 10 mM NH_4NO_3 CONTAINS 1 mmol NH_4NO_3

$$NH_4NO_3 \longrightarrow NH_4^+ + NO_3^-$$

$$\therefore \quad 1 \text{ mmol } NH_4NO_3 \longrightarrow \text{mmol } NH_4^+ + \text{mmol } NO_3^-$$

(2) 1000 mL 10 mM KNO_3 CONTAINS 10 mmol KNO_3

$\therefore$ 100 mL 10 mM KNO_3 CONTAINS 1 mmol KNO_3

$$KNO_3 \longrightarrow K^+ + NO_3^-$$

$\therefore$ 1 mmol $KNO_3 \longrightarrow$ 1 mmol K^+ + m mol NO_3^-

(3) 1000 mL 10 mM $Ca(NO_3)_2$ CONTAINS 10 mmol $Ca(NO_3)_2$

$\therefore$ 100 mL 10 mM $Ca(NO_3)_2$ CONTAINS 1 mmol $Ca(NO_3)_2$

$$Ca(NO_3)_2 \longrightarrow Ca^{2+} + 2NO_3^-$$

$\therefore$ 1 mmol $Ca(NO_3)_2 \longrightarrow$ 1 mmol Ca^{2+} + 2 mmol NO_3^-

(4) (1) + (2) +(3):

(1)	1 mmol NH_4^+	+ 1 mmol NO_3^-
(2)	1 mmol K^+	+ 1 mmol NO_3^-
(3)	1 mmol Ca^{2+}	+ 2 mmol NO_3^-
		= 4 mmolNO_3^-

EXERCISES

1. A published culture medium has a stated concentration of sucrose in the medium of 60 mM. How many grams of sucrose per litre do you require. Molwt of sucrose = 342.30.

2. If we use 4.108 g/L of sucrose what is the molarity of sucrose in this solution.

3. A published culture medium which you want to prepare states that 10 mg $ZnSO_4$ is used but you can get only $ZnSO_4, 7H_2O$. What would you do?
   Molwt of $ZnSO_4$ = 161.446.
   Molwt of $ZNSO_4, 7H_2O$ = 287.558.

4. How much of the following compounds need to be included in one litre of medium to make a concentration of 50 µM $MnSO_4$.

		Mwt
(1)	$MnSO_4$	151
(2)	$MnSO_4, H_2O$	169
(3)	$MnSO_4, 4H_2O$	223
(4)	$MnSO_4, 6H_2O$	259
(5)	$MnSO_4, 7H_2O$	277

5. You have a stock solution containing 1000 µM BAP. You wish to prepare a litre each of 3 different media containing 2, 5 and 10 µM of BAP. What volume (mL) of stock solution would you use for each medium?

APPENDIX 4

USE OF UV LAMP

UV irradiation poses some serious health risks. One should never look at a live UV tube with the naked eye. UV burns to the eye (actinic keratitis) are very painful, although not normally of lasting effect. A glass barrier, between the eyes and the UV lamp, provides complete protection. Also, UV irradiation can produce irritation to unprotected skin; so avoid placing the hands in the transfer chamber when the lamp is in use. Another problem is the formation of ozone (O_3) resulting from the photochemical reaction with atmospheric oxygen. This explosive gas is a powerful oxidizing agent, and high concentrations can cause severe irritation to the respiratory tract and eyes. The UV lamp should never be left on for long periods of time with the transfer chamber sealed.

Never use hypochlorite or other inorganic chloride preparations in the presence of UV irradiation. The resulting release of free chlorine gas is a serious health hazard.

APPENDIX 5

GEL CONSISTENCY

If the gel consistency is too soft it could be due to the following reasons:

1. The pressure of the autoclave may have been set too high.
2. The temperature of the autoclave may have been set too high.
3. The time of autoclaving may be too long.
4. Lack of pressure valve control: in this case the pressure stays high for much longer than anticipated.
5. The pH of the medium is incorrectly set too low. pH below 4 will produce a runny gel.
6. Gel concentration too low.
7. Type of sugar used, e.g. fructose makes a softer gel.
8. Combination of all above.

APPENDIX 6

ACTIVATED CHARCOAL

Activated or decolorizing carbon becomes deactivated with time and in order to have uniformly activated material one should carry out re-activation in the laboratory as follows:

For 20 g of charcoal to be reactivated use 250-300 mL of 2 N HCl (Handle the acid carefully.) in a 500-600 mL beaker with glass boiling chips and heat to boiling with continuous stirring.

Boil for 3-4 hours in a fume hood.

Cool to room temperature and wash with distilled water until Cl^- free on a Buchner funnel. Warm distilled water (50-60°C) removes Cl^- more easily. Ten to twenty litres of water may be necessary for washing. When using nominally activated charcoal that has been activated sometime earlier, heat first at 110°C for an hour or so. Heating drives off any oxygen that occupies the sites available for absorption.

The charcoal can thus be stored in a desiccator and used as necessary.

APPENDIX 7

WATER POTENTIAL IN VITRO

The water potential within a culture vessel is made up of two separate components:

(i) the osmotic potential
(ii) the matric potential

The osmotic potential is attributed to the presence of solutes in the water, whereby an increase in carbohydrate concentration will cause the osmotic potential to become more negative.

The matric potential is attributed to the presence of insoluble substances in the medium, an increase in agar concentration causes the matric potential to become more negative.

Increasing the agar concentration in a culture medium reduces the availability of water to the plant material and lowers the relative humidity. However increasing the sugar concentration has no effect on the relative humidity within a culture vessel.

APPENDIX 8

PLANT GROWTH REGULATORS IN VITRO

The following are lists of Auxins, Cytokinins and other plant growth regulators used in tissue culture.

AUXINS

p-CHLOROPHENOXYACETIC ACID (4-CPA; CPA)
- autoclavable
- working conc.:5.0-50.0 µM

2,4-DICHLOROPHENOXYACETIC ACID (2,4-D)
- autoclavable
- working conc.:0.05-25.0 µM

INDOLE-3-ACETIC ACID (IAA)
- some loss of activity may occur during autoclaving, compensate by increasing the component concentration, for critical experimentation should be filter sterilized.
- light sensitive
- working conc.:5.0-15.0 µM

INDOLE-3-ACETYL-L-ALANINE (IAA-L-Alanine)
- filter sterilization only
- working conc.:0.05-25.0 µM

INDOLE-3-ACETYL-L-ASPARTIC ACID (IAA-L-Aspartic acid)
- filter sterilization only
- working conc.:0.05-25.0 µM

INDOLE-3-ACETYLGLYCINE (IAA-Glycine)
- filter sterilization only
- working conc.:0.05-25.0 µM

INDOLE-3-ACETYL-L-PHENYLALANINE (IAA-L-Phenylalanine)
- filter sterilization only
- working conc.:0.05-25.0 µM

INDOLE-3-BUTYRIC ACID = 4-[3-INDOLYL]BUTYRIC ACID (IBA)
- autoclavable, some loss of activity may occur during autoclaving, compensate by increasing the component concentration.
- slightly light sensitive
- working conc.:0.5-50.0 µM

1-NAPHTHALENEACETIC ACID (NAA)
- autoclavable
- working conc.:0.5-50.0 µM

β-NAPHTHOXYACETIC ACID (NOA)
- autoclavable
- working conc.:0.05-50.0 µM

Auxins can be dissolved in a few drops of concentrated alcohol. Add distilled water to make up to the required volume.

CYTOKININS

ADENINE
- autoclavable
- working conc.:250-1250 µM

ADENINE HEMISULPHATE
- autoclavable
- working conc.:250-1250 µM

6-BENZYLAMINOPURINE = N^6-BENZYLADENINE (BAP or BA)
- autoclavable
- working conc.:0.5-25 µM

6-BENZYLAMINOPURINE RIBOSIDE = N^6-BENZYLADENOSINE
- filter sterilization
- working conc.:0.05-25 µM

N-BENZYL-9-(2-TETRAHYDROPYRAN-YL)ADENINE = 6-BENZYLAMINO-9-[2-TETRAHYDROPYRANYL]9H PURINE (BPA)
- some loss of activity may occur during autoclaving
- working conc.:0.05-25 µM

6-(γ,γ DIMETHYLALLYLAMINO)-PURINE = N^6-[2-ISOPENTENYL]ADENINE (2IP)
- some loss of activity may occur during autoclaving
- working conc.:5.0-150 µM

6-(γ, γ DIMETHYLALLYLAMINO)-PURINE RIBOSIDE = N^6γ,γ DIMETHYLALLYLADENOSINE = N^6-[2-ISOPENTENYL]ADENOSINE
- filter sterilization
- working conc.:5.0-150 µM

KINETINE = 6-FURFURYLAMINOPURINE
- autoclavable
- working conc.:0.5-25 µM

KINETINE RIBOSIDE = 6-FURFURYLAMINOPURINE RIBOSIDE = N^6-FURFURYLADENOSINE
- filter sterilization
- working conc.:0.05-25 µM

ZEATIN = 6-[4-HYDROXY-3-METHYL-BUT-2-ENYLAMINO] PURINE trans and mixed isomers
- some loss of activity may occur during autoclaving
- working conc.:0.05-25 µM

t-ZEATIN RIBOSIDE
- filter sterilization
- working conc.:0.05-25 µM

Cytokinins can be dissolved in a few drops of 1N HCl. Add distilled water to make up to the required volume.

OTHER PLANT GROWTH REGULATORS USED IN TISSUE CULTURE

ABSCISIC ACID
- some loss of activity may occur during autoclaving
- working conc.:0.5-50 µM

trans-CINNAMIC ACID = β-PHENYLACRYLIC ACID = 3-PHENYL PROPENOIC ACID
- some loss of activity may occur during autoclaving
- working conc.:0.5-50 µM

1,3-DIPHENYLUREA (CARBANILIDE)
- filter sterilization
- working conc.:0.5-50 µM

GIBBERELLIC ACID (GA_3)
- some loss of activity may occur during autoclaving
- working conc.:0.05-25 µM

3-OXO-2-(2'-PENTENYL)-CYCLOPEN-TANEACETIC ACID (JASMONIC ACID)
- autoclavable
- working conc.:0.05-15 µM

N-PHENYL-N'-(2-CHLORO-4-PYRIDYLE) UREA = (4PU-30)
- some loss of activity may occur during autoclaving
- working conc.:0.1-25 µM

N-PHENYL-N'1,2,3-THIDIAZOL-5-YL UREA = TDZ
- some loss of activity may occur during autoclaving
- working conc.:0.1-25 µM

PHLOROGLUCINOL = 1,3,5-TRIHYDROXYBENZENE
- some loss of activity may occur during autoclaving
- working conc.:5.0-50 µM

SUCCINIC ACID 2,2-DIMETHYL HYDRAZIDE (DAMINOZIDE)
- some loss of activity may occur during autoclaving
- working conc.:5.0-50 µM

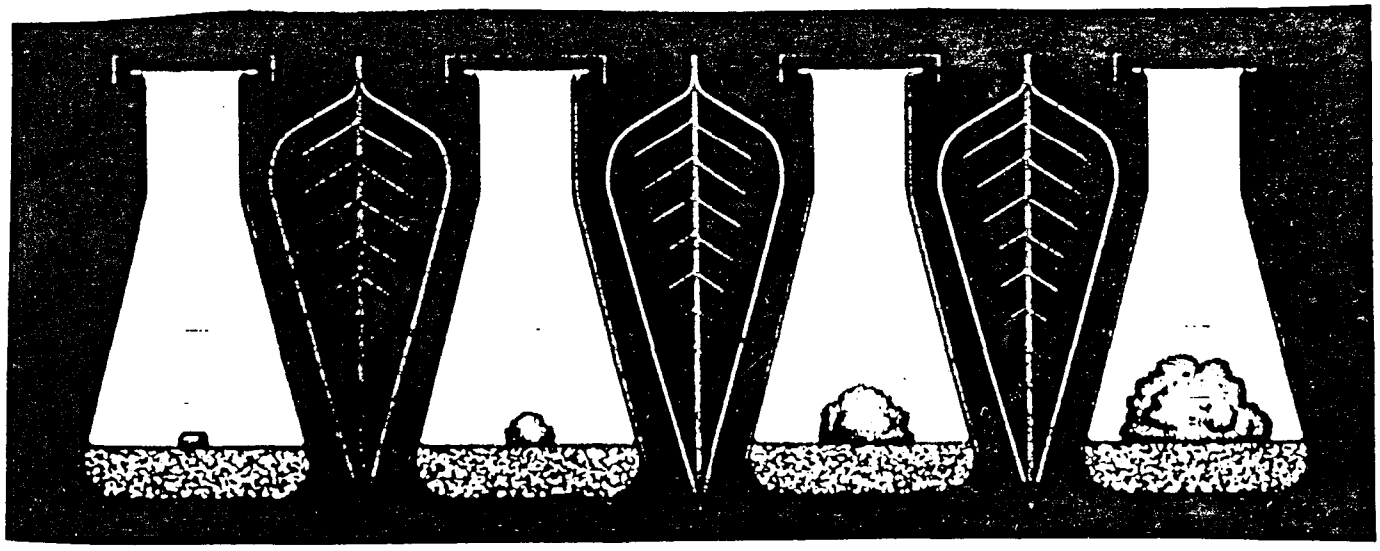
Prepare stock solutions of 1000 µM for all the chemicals. Add appropriate quantity of the required stock solution to the medium to obtain the desired concentration. Use the following equation to work out the concentrations in the medium:

$C_S \times V_S = C_m \times V_m$
C = concentration
V = volume
S = stock
M = medium

APPENDIX 9

PREPARATION OF TUNGSTEN WIRE NEEDLES

For many dissections, a pair of tungsten wire needles can be used in a scissor-like motion. Tungsten wire can be obtained in different gauges, and a series of needles from narrow to thick gauge (400 μm to 1000 μm) can be prepared for dissections of varying degrees of thickness. Break off approximately 5 cm of tungsten wire, place one end in a needle holder. The other end of the tungsten wire is then sharpened by dipping it into molten and near boiling sodium nitrite ($NaNO_2$); the end of the wire incandesces during this operation. (Extreme caution must be exercised during this operation - work in a fume hood.) Inspect the end of the tungsten wire needle under a stereomicroscope and repeat the $NaNO_2$ dip until a sharp point has been achieved; with care and appropriate use, the points will remain sharp for long periods of use.



APPENDIX 10

ROUGH AND READY METHOD FOR PLANT TISSUE CULTURE

Method I

The following method is used by people who have no access to a research laboratory. All the ingredients can be purchased using a supermarket, chemist and a health food shop. The recipe is as follows:

1. Tap water: 2 cups
2. Table sugar: $^1/_4$ cup
3. Stock: $^1/_2$ tablespoon all purpose 10:10:10 water soluble fertilizer in 1 L H_2O: 1 cup of stock
4. Inositol tablets (500 mg): $^1/_2$ tablet
5. Vitamin tablet with thiamine: $^1/_2$ tablet
6. Agar flakes: 4 tablespoons

Boil until the agar has dissolved, stirring continuously. Dispense into empty babyfood jars or any other suitable jars, using a ladle, so that the medium is about 2 cm deep. Cover and process in a pressure cooker. Cook for 15 minutes after the pressure is reached (this will be achieved when the pressure valve starts letting steam out). Wrap forceps and razor blades in Alfoil and sterilize in the pressure cooker at the same time. Sterile water is needed for rinsing plant material and sterile paper towelling to be used as a clean surface to work on. The manipulation can be accomplished within the fold of a towel while the folded half forms a protective hood which is effective against air borne contamination. When the operation is completed, the towel can be discarded and a new sterile surface selected from the sterile supply.

All plant materials can be sterilized in diluted white king ($^1/_4$ cup of white king + $^3/_4$ cup of water + 1 drop of detergent - detergent acts as surfactant). Put plant pieces in a jar containing the bleach for 10 - 20 minutes. Agitate frequently. Discard the chlorine solution and rinse plant pieces with sterile water. Small pieces, 2-3 cm long with a few leaves can be cut and transferred to agar medium. If the leaves are too large either remove them or cut them to
$^1/_3$ - $^1/_2$ the size. Store jars at room temperature away from direct sunlight.

Seeds can be surface sterilized as above and germinated under aseptic conditions.

All instruments can be dipped in methylated spirit and flamed on a candle.

ASEPTIC SEED GERMINATION

Rough and Ready Method II

Select sound seeds and surface sterilize them as follows:

1. Place seeds in a glass jar containing dilute methylated spirit ($^1/_2$ cup of methylated spirit + $^1/_2$ cup of water). Agitate for 30 seconds.

2. Discard the alcohol and cover seeds with dilute white king ($^1/_4$ cup of domestic bleach + $^3/_4$ cup of water + 1 drop of detergent), for 15 minutes.

3. Drain the bleach. Rinse seeds in sterile water once and soak in sterile water overnight.* Cover jars with their lids or gladwrap and store in a clean place.

4. Next day, transfer 5 seeds to each baby food jar containing medium (follow instructions from "Rough and Ready Method for Plant Tissue Culture I").

5. Store some of the jars in a dark cupboard and some in light (Temp. 25±2°C). Compare the rate of germination and subsequent growth of the seedlings after a week and again 2 weeks later.

* If using Acacia or Sturt's Desert Pea seeds, apply boiling water before soaking seeds overnight. This will loosen up the seed coat and aid germination.

PRODUCTION OF CALLUS/SHOOT/ROOT

Rough and Ready Method III

1 cm segments of hypocotyl or the shoot tip from seedlings germinated as in previous sheet ("Aseptic seed germination") can be grown to produce callus/shoot/root using the following medium:

To the basic medium as in "Rough and Ready Sheet" add $^1/_2$ cup of coconut milk and $^1/_2$ teaspoon of malt. Replacing the coconut milk with $^1/_2$ cup of green tomato puree or $^1/_2$ cup of freshly squeezed orange juice may produce different responses. Ensure that the pH of medium is always between 5 and 6 using narrow range pH tape or pH meter.

APPENDIX 11

CULTURE CHECK LIST

1.	Green healthy growth.	Suspect contamination.	SEE POINT '5'
2.	Green healthy growth.	No evidence of contamination.	SEE POINT '6'
3.	Pale unhealthy growth.	Suspect contamination.	SEE POINT '7'
4.	Pale unhealthy growth.	No evidence of contamination.	SEE POINT '7'
5.	Carry out sterility test.	No contamination. Contamination.	SEE POINT '6' SEE POINT '9'
6.	Monitor progress of cultures (carry out sterility test, if desired)		
7.	Carry out sterility test.	No contamination. Contamination	SEE POINT '8' SEE POINT '9'
8.	Investigate media formulation.		SEE POINT '10'
9.	Commence disinfestation programme. If there is difficulty obtaining further stock plants or discard contaminated cultures and re-initiate		SEE POINT '15'
10.	Suspect incorrect media formulation. No evidence of incorrect media formulation.		SEE POINT '11' SEE POINT '14'
11.	Check for deficiency/toxicity symptoms.	No symptoms Symptoms	SEE POINT '12' SEE POINT '13'
12.	Remake stock solutions and media, taking care over quality assurance.		
13.	Deficiency symptoms:		

Nitrogen	Leaves overall yellow-green colour with older leaves more severely affected; may turn completely yellow and abscise. Leaves generally stunted, and internode and stem diameter are reduced.
Phosphorus	Overall stunting of leaves and shoot growth. Later the older leaves may drop off without turning yellow. Root development is reduced.
Potassium	Stunted growth. Tip and marginal yellowing, browning, and necrosis of older, lower leaves.
Calcium	Young leaves are distorted. Later, the edges of leaves turn yellow and brown. Discoloured spots on the leaves may coalesce into large necrotic blotches.

Magnesium	Shows up primarily on lower, older leaves. Chlorosis of the older leaves begins in interveinal areas and progresses to necrosis. Small spots initially, but later larger and larger areas. Overall growth is stunted.
Sulphur	Slight interveinal chlorosis of the young leaves, initially. May progress to an overall light yellow-green colouration of entire new growth.
Iron	Interveinal chlorosis, initially, of the young leaves, main veins remain green. If deficiency continues, the new formed leaves may remain very small - eventually pale yellow or almost white. May not be shortage of iron - but interference in uptake caused by - poor aeration, high soluble salt levels, excessively high or low temperature, high concentrations of manganese, zinc or phosphorus.
Copper	First symptoms include distorted young leaves with yellow tips that later become necrotic. Growing point dies, and short, stunted lateral shoots then develop.
Zinc	Almost identical to those caused by copper deficiency, except that in zinc deficiency, the lateral shoots that develop after meristem dies are severely stunted.
Boron	Necrosis of growing point, followed by development of lateral shoots and death of the growing point, followed by further development of more lateral shoots, i.e. witches broom type of symptom. [Note - specific balance between calcium/boron essential.]
Molybdenum	Resemble those of moisture stress - namely browning and necrosis of the tips and edges of leaves.

TOXICITY SYMPTOMS:

Nitrogen	Stunted growth and dark green foliage. Also osmotic stress - marginal necrosis of the leaves.
Phosphorus	May interfere with availability of copper, iron, zinc and calcium.
Potassium	Similar to toxicity symptoms caused by high total soluble salt levels: chlorosis and marginal leaf necrosis, root loss, and wilting of soft succulent shoots.
Sulphur	Similar to toxicity symptoms caused by high total soluble salt levels: chlorosis and marginal leaf necrosis, root loss, and wilting of soft succulent shoots.
Calcium	Can cause adverse effects on the availability and uptake of other essential mineral elements, e.g. iron and phosphorus.

Magnesium	Similar to calcium.
Manganese	Can show small black spots in the interveinal areas. Interveinal chlorosis of the young leaves, typical of iron deficiency symptoms, can also occur because a balance between iron and manganese is needed for normal growth.
Iron	May cause deficiency symptoms of copper, manganese, or zinc because of the interrelationships between these nutrients and iron.
Copper	Often results in leaf abscission followed by excessive leaf drop.
Boron	Levels only slightly above normal can quickly cause marginal browning and necrosis of older leaves. Boron toxicity symptoms may be confused with magnesium or calcium deficiency symptoms.
Zinc	Initially, there is appearance of light green, transparent, water-soaked areas along the veins of the leaves. Later, the rest of the leaf turns yellow, then brown. Affected areas abscise irregularly. Foliage drops only after the leaves have turned completely brown.

IDENTIFY PROBLEM AND RETURN TO POINT '2':

14. Check the following parameters:-

Temperature stress Moisture stress	Marginal necrosis of stems and leaves.
Oxygen deficiency	Yellowing of main veins followed by interveinal necrosis of young foliage, and a major leaf drop of more mature leaves.
Ethylene toxicity	Curling of the youngest leaves, and yellowing along the principal veins of the older leaves. Stem elongation is slowed. Some decrease in apical dominance often resulting in the growth of more than the normal number of axillary buds.
Plant volatiles/Plasticizers	Paints containing xylene, naphthalene and mineral spirit cause necrosis and distortion, and lower leaves readily abscise.

Identify problem and make necessary rectifications.

15. Sterilize material in 0.1-0.2% sodium hypochlorite. Rinse in sterile water. Dissect material almost to final inoculum size - discard unwanted leaves, roots etc.

Second sterilization in 0.1% sodium hypochlorite ± rinse. Dissect further, if necessary.

Inoculate onto basic medium with low hormonal level and monitor closely.

Some Principles of Visual Diagnosis of Nutritional Disorders[a]

Plant part	Prevailing symptom		Disorder
Old and mature leaf blades	Chlorosis	Uniform	*Deficiency*
		Interveinal or blothched	N (S)
	Necrosis	Tip and marginal scorch	Mg (Mn)
		Interveinal	K
			Mg (Mn)
Young leaf blades and apex	Chlorosis	Uniform	Fe (S)
		Interveinal or blotched	Zn(Mn)
	Necrosis (chlorosis)		Ca, B, Cu
	Deformations		Mo (Zn, B)
			Toxicity
Old and mature leaf blades	Necrosis	Spots	Mn (B)
		Tip and marginal scorch	B, salt (spray injury)
	Chlorosis, necrosis		Nonspecific toxicity

[a] Letters in parentheses indicate that symptoms are variable

APPENDIX 12

INTERCONVERSIONS OF SOME UNITS USED IN TISSUE CULTURE

Tissue culture equipment and formulae for media are measured or expressed in metric units. The following units are defined in metric and imperial terms to allow interconversions if necessary.

Volume

1000 millilitres (mL) = 1 litre (L)
1 gallon = 4.55 L
1 fluid ounce (imp) = 28.35 mL
1 cubic centimetre (cm) = 0.06 cubic inches

Concentration

1000 milligrams (mg) = 1 gram (g)
1 mg L^{-1} = 1 mg per litre = 1 part per million (ppm)
1 mole = the molecular weight of a compound expressed in grams
1 molar solution = gram molecular weight (one mole) in a litre of water

Length

1 inch (in) = 25.4 millimetres (mm)
1 centimeter (cm) = 0.39 inches

Light

1 lux = 0.093 foot candles
1 foot candle = 10.8 lux
1 micro Einstein per square meter per second ($\mu E\ m^{-2}\ sec^{-1}$) = 54 lux
1 μ mole $m^{-2}\ sec^{-1}$ = 1μ Einstein $m^{-2}\ sec^{-1}$ to measure PPFD

Weight

1000 milligrams (mg) = 1 kilogram (kg)
1 kilogram = 2.2 pounds
1 ounce (imp) = 28.35 grams

Pressure

1 kilopascal (kPa) = 0.145 pounds per square inch (psi)
1 psi = 6.89 kPa
1 bar = 100 kPa

APPENDIX 13

INSTRUCTION FOR ACCLIMATIZATION OF TISSUE-CULTURE GROWN PLANTS

Work out of direct sunlight

1. Gently remove plants from gel and wash them in lukewarm water, ensure that all gel (agar) particles are completely removed.

2. Transfer rooted explants to a pot containing peat: vermiculite: sand (1:1:1). Pots should be well watered prior to use and drained.

3. Spray plants with water regularly using a hand spray.

4. Transfer plants either to a humid chamber or under a mister with reduced light intensity using a shade cloth (30% day light) at 25°C.

5. Gradually over the next four weeks remove the lid of chamber (or reduce frequency of misting) and increase the light intensity. From week 2 water plants with a weak solution of Aquasol (N:P:K:23:4:18 0.25 g/L) twice a week.

6. On week four transfer plants from under the mister or the chamber to the bench in a glass house.

7. On week five, when you can see the roots emerging from the bottom of the propagating pots, remove plants and transfer them to large pots containing peat: vermiculite: sand (1:1:3) with complete fertilizer.

N.B. No spraying with fungicide or pesticide should be carried out during acclimatization. Diseased or affected plants should be removed quickly and the area be swabed with diluted chlorine solution.

APPENDIX 14

ATOMIC WEIGHT OF ELEMENTS USED IN TISSUE CULTURE

Element	*Symbol*	*Atomic Weight*
Aluminium	Al	26.98
Boron	B	10.811
Calcium	Ca	40.08
Carbon	C	12.01115
Chlorine	Cl	35.453
Cobalt	Co	58.9332
Copper	Cu	63.54
Hydrogen	H	1.00797
Iodine	I	126.9044
Iron	Fe	55.847
Magnesium	Mg	24.312
Manganese	Mn	54.938
Molybdenum	Mo	95.94
Nitrogen	N	14.0067
Oxygen	O	15.9994
Phosphorus	P	30.9738
Potassium	K	39.102
Sodium	Na	22.9898
Sulfur	S	32.064
Zinc	Zn	65.37

APPENDIX 15

PREPARATION OF COCONUT MILK FOR INCLUSION IN MEDIA

Ripe coconuts may be purchased from vegetable shops already dehusked. Each coconut contains 100-200 mL of liquid endosperm. Three micropyles (eyes) are located at one end of the coconut, one of which is composed of soft tissue, easily removed with a cork borer or punctured using a screw driver. The liquid will pour more easily if an air vent is created by puncturing a second micropyle with an electric drill or a hammer and nail. Drain each coconut separately because some of the coconuts may contain fermented milk. This milk which is easily identified by its odor and colour should be discarded.

The collected milk should pass through the following steps prior to use in a medium:-

1. Filter through several layers of cheese cloth.
2. Boil the filtrate for approximately 10 minutes in order to precipitate the proteins.
3. Cool to room temperature
4. Decant and filter the supernatant through a filter paper. Buchner funnels with filter papers may be used at this stage.
5. To 1 litre of nutrient medium add 100-150 mL of coconut milk.

Any unused coconut milk can be frozen for use at a later date. Melting and refreezing do not seem to affect the cytokinin-like properties of the substances present in the liquid endosperm.

APPENDIX 16

MEDIA FOR PROPAGATION OF AUSTRALIAN NATIVE PLANTS

The following table provides information on media used at multiplication and rooting stages for 21 Australian Native Plant species. These media produced the best response in our laboratory and should be used as a starting point for those who wish to use them.

Species	Family	Multiplication medium	Rooting medium / pH
Acacia ligulata	Mimosaceae	de Fossard's MZZM	de Fossard's MZZM / 5.5
Anigozanthos bicolor	Haemodoraceae	M & S + 2µM BAP + 0.5µM NAA	M & S + 5µM IBA / 5.8
Chamelaucium uncinatum	Myrtaceae	de Fossard's $^3/_4$ strength high Macro, Micro and Organics + 0.5µM IBA + 1µM BAP + 1µM Kin Sucrose 90mM	$^1/_2$ M & S + 9.0µM IBA + Sucrose 120mM / 5.8
Correa decumbens	Rutaceae	de Fossard's Medium Macro, Micro and Organics + 0.5µM BAP+ 0.5µM Kin	de Fossard's Medium Macro, Micro and Organics + 2µM IBA + 2µM NOA / 4.0
Dampiera diversifolia	Goodeniaceae	de Fossard's Medium Macro, Micro and Organics + 0.5µM BAP+ 0.5µM Kin	de Fossard's Medium Macro, Micro and Organics + 0.5µM IBA + 0.5µM NAA /5.5
Davidsonia pruriens	Davidsoniaceae	M & S + 0.5µM BAP	M & S + 1µM IBA/5.5
Eremophila laanii	Myoporaceae	de Fossard's Medium Macro, Micro and Organics + 1µM BAP + 1µM Kin	de Fossard's Medium Macro, Micro and Organics + 1µM BAP + 1µM Kin / 5.5
Grevillea biternata	Proteaceae	de Fossard's Medium Macro, Micro and Organics + 1µM BAP + 1µM Kin	de Fossard's Medium Macro, Micro and Organics + 0.5µM IBA + 0.5µM NOA / 5.5
Grevilleax Robyn Gordon	Proteaceae	de Fossard's Medium Macro, Micro and Organics + 1µM BAP + 1µM Kin	de Fossard's Medium Macro, Micro and Organics + 5µM NAA / 5.5
Kreysigia multiflora	Liliaceae	de Fossard's High Macro, Micro and Organics + 4µM BAP + 0.5µM NAA	de Fossard's Medium Macro, Micro and Organics + 10µM IBA 120 mM sucrose/ 5.5

Species	Family	Multiplication medium	Rooting medium / pH
Lechenaultia formosa	Goodeniaceae	M & S + 0.5µM BAP	M & S + 2µM NAA / 5.9
Macropedia fuliginosa.	Haemodoraceae	M & S + 2µM BAP + 0.5µM NAA	M & S + 3µM IBA / 5.5
Melaleuca alternifolia	Myrtaceae	M & S + 2µM BAP	$^1/_2$ strength M & S / 5.8
Ptilotus exaltatus	Amaranthaceae	M & S + 0.5µM BAP	M & S + 1µM NOA then transfer to Basal / 5.5
Prostanthera calycina	Lamiaceae	de Fossard's Medium Macro, Micro and Organic 1µM BAP + 1/M Kinetin	de Fossard's Medium Macro, Micro and Organics + 0.5µM IBA/5.5
Prostanthera eurybioides	Lamiaceae	de Fossard's Medium Macro, Micro and Organics + 1µM BAP + 1µM Kin	de Fossard's Medium Macro, Micro and Organics + 0.05µM IBA + 0.05µM NAA / 5.5
Prostanthera rotundifolia	Lamiaceae	de Fossard's Medium Macro, Micro and Organics + 1µM BAP + 1µM Kin	de Fossard's Medium Macro, Micro and Organics + 2µM IBA + 2µM NOA / 5.5
Prostanthera striatiflora	Lamiaceae	de Fossard's Medium Macro, Micro and Organics + 1µM BAP + 1µM Kin	de Fossard's Medium Macro, Micro and Organics + 0.5µM IBA + 0.5µM NOA / 4.0
Rhagodia spinescens	Chenopodiaceae	de Fossard's Medium Macro, Micro and Organics + 1µM BAP	de Fossard's Medium Macro, Micro and Organics + 0.5µM IBA + 0.5µM NAA / 5.5
Schefflera actinophylla	Araliaceae	de Fossard's Medium Macro, Micro and Organics + 50µM Kinetin + 10µM NAA	Root on multiplication medium / 5.5
Stirlingia latifolia	Proteaceae	de Fossard's Medium Macro, Micro and Organics + 2µM BAP	de Fossard's Low Macro, Micro and Organics i.e. LZZL/ 5.5 .

IN VITRO FERN SPORE CULTURE

PREPARATION OF FERN MEDIUM

Preparation of stock solutions:

A. NH_4NO_3 = 40.025 g
KNO_3 = 50.55 g
$MgSO_4.7H_2O$ = 18.487 g

Dissolve in boiling water. When cool bring volume to 1 litre.

B. NaH_2PO_4 = 7.8 g
Dissolve in hot water. When cool bring volume to 1 litre.

C. 75 mL of 1N $CaCl_2$ solution. Make-up to 1 litre.

D. FeNaEDTA = 0.918 g
Dissolve in water and bring to 1 litre.

E. Micro-elements Stock.

First make the following 3 solutions:

(1)	$CuSO_4.5H_2O$	=	0.0624 g	in 1 litre water
(2)	$Na_2MoO_4.2H_2O$	=	0.0605 g	in 1 litre water
(3)	$CoCl_2.6H_2O$	=	0.0595 g	in 1 litre water

Then

H_3BO_3	=	0.232 g
$MnSO_4.H_2O$	=	0.423 g
$ZnSO_4.7H_2O$	=	0.288 g
KI	=	0.021 g

plus

10 mL $CuSO_4.5H_2O$ solution (1) above
100 mL $Na_2MoO_4.2H_2O$ solution (2)
10 mL $CoCl_2.6H_2O$ solution (3)

Make to final volume of 1 litre.

Preparation of the medium.

Take 5 mL each of stocks A,B,C,D,E
Add 225 mL water
+ 5.135 g sucrose

Adjust pH = 5.5
Dispense 5 mL/tube
Dissolve 4 g agar in 250 mL of water and dispense 5 mL to each tube.
Cap tubes and autoclave for 20 min.

REMOVAL OF SPORE FROM FERN FROND

1. Scrape sori containing spores from the frond into a dry petri dish.
2. Place uncovered dish into a desiccator containing a layer of silica gel (or other desiccant material).
3. When the spores are released, sift through a fine screen (100-150 mesh).
4. Place spores in an air-tight container, label and store in the refrigerator (about 4°C).

STERILIZATION OF SPORES

1. Shake spores in a centrifuge tube using H_2O and a drop of wetting agent (e.g. Triton or Tween 20).
2. Centrifuge to settle the spores to the bottom of the tube. Discard the water without disturbing the spores.
3. Add 10 mL of sterile water, cap, shake and leave at room temperature for 24 hours.
4. Centrifuge again, and discard the water.
5. Add 10 mL of 1% Sodium hypochlorite (NaOCl) to the tube and leave for 5 minutes. Centrifuge and discard the solution again.
6. Rinse 3 times with sterile water, centrifuge each time to settle spores to the bottom.
7. Aseptically transfer spores onto the culture medium using a pipette or a microbiological loop.

APPENDIX 17

PLANT SECTIONS AND STAINS

In a tissue culture laboratory it is sometimes necessary to check the anatomy of in vitro grown plants. These notes provide much useful information about the cutting and staining of plant sections for anatomical studies.

TYPES OF SECTIONS

The internal structure of plants is usually studied by examining very thin slices, or **sections** of material often **stained** with dyes which differentially stain various parts of the cells. These sections can be cut by hand using a razor blade or mechanically using a microtome. Plant material can be sectioned fresh or preserved or prepared for very thin sectioning using a microtome by embedding in wax or plastic. The preparation of sections for examination with the electron microscope (giving up to x 100,000 magnification) requires special techniques and ultra-thin sections.

Many plant organs are approximately cylindrical, e.g. roots and stems and the usual planes in which sections are cut are:

(i) **Transverse section** (T.S.) - also occasionally called a cross-section or X.S. where the cutting plane is at right angles to the longitudinal axis.

(ii) **Radial longitudinal section** (R.L.S.) - i.e. a longitudinal section which is also on a radius of the cylinder. Compare with a sagittal section in animal biology.

(iii) **Tangential longitudinal section** (T.L.S.) - i.e. a longitudinal section which is also on a tangential plane of the cylinder.

Knowledge of the plant from which the material has been cut is essential for **interpretation** of the structures observed. To understand the structure of a whole organ, it is necessary to **reconstruct a three-dimensional picture** from the various sectional views. The fourth dimension of **time** must also be considered when studying the **growth** and **development** of plant structures.

CONSTRUCTION MATERIALS IN PLANT CELLS - MICROCHEMICAL TESTS

1. Cellulose. This is the basic constituent of the cell wall in higher plants (and also many algae and some fungi). Chemically cellulose is a long-chain polysaccharides made up of D-glucose (6 carbon with a ß 1-4 linkage). In the **primary cell wall** these long chain molecules lie parallel and are bonded together in groups of up to 200 to form crystalline **micelles.** There are also other materials - **hemicellulose,** pectic substances and small amounts of **protein** in the primary wall and the loose network formed allows for wall extension by more cellulose being laid down between existing micelles.

Cellulose is of limited elasticity, is relatively rigid, and is also relatively permeable to many substances, e.g. water will pass through cellulose cell walls fairly rapidly (filter paper is a very pure form of cellulose). The enzymes capable of breaking down cellulose occur mainly in lower organisms, e.g. fungi, bacteria and protozoans but do not usually occur in higher plants or animals.

Test for Cellulose: Soak for a few moments in iodine, then mount in a drop of 50% sulphuric acid. A blue colouration will occur. Cellulose stains a bluish purple with toluidine blue.

2. Lignin. Chemically, lignin is a non-crystalline, high molecular weight substance representing the condensation product of aromatic alcohols but the precise chemistry is not fully understood. It is deposited inside the cellulose cell walls of certain plant cells, e.g. sclerenchyma fibres, xylem vessels and xylem tracheids. This renders them strong, rigid, inelastic, and more or less impermeable to water. Lignin forms 25-30% of the weight of wood.

Usually heavily lignified cells have no nucleus, or protoplasm, and therefore carry on no metabolism and are not osmotically active.

Test for Lignin:

(i) Lignin will give a red colour if stained with phloroglucin + HCl.

(ii) It will give a yellow colour if stained with aniline sulphate + H_2SO_4 or aniline chloride + HCl.

(iii) Lignin stains blue with toluidine blue.

3. Suberin and Cutin. These are fatty or waxy substances, which are very impermeable to water. Cutin is secreted by epidermal cells to form a waxy, non-cellular layer of cuticle, which is broken only by stomates. The main function of this relatively waterproof layer is to prevent excessive water loss. Older parts of plants may have a layer of cork, and suberin is present in the walls of the cork cells making them impervious to water, except where a lenticel occurs.

Test for suberin and cutin: Stain with Sudan III, wash in 50% alcohol, and mount in glycerine. Subserized or cutinized walls will stain red.

4. Pectic Substances. These are acid polysaccharide carbohydrates derived partly from 6 carbon sugar acids. They form **gels** under certain conditions (and hence are often used in the manufacture of jams and jellies). In the formation of new cells, pectic material is laid down to form the **middle lamella** which forms the junction between the primary cell walls of adjacent cells.

Test for pectic substances: Ruthenium red will stain the middle lamella a dark red.

MATERIALS PRESENT IN PLANT CELLS

1. Starch is one of the main non-structural carbohydrates in the cells found inside chloroplasts where it is formed from photosynthesis in leaves or in **leucoplasts** (amyloplasts) of storage organs e.g. potato tubers, where it is synthesized following sugar translocation to these regions. Chemically, starch is a polysaccharide with two components **amylose** and **amylopectin** both of which are made up of -D-glucose units (6 carbon) connected in α1-4 linkages to give long chains. The amylose chains are unbranched, and the molecule is built up of about 200-1,000 glucose units. The amylopectin molecules are built up of about 2,000 up to 200,000 glucose units, and the chains are branched. These differences between amylose and amylopectin result in slightly different types of starch being formed by different plants according to the differing ratios of the two components.

Starch can be broken by enzyme action into sugars or sugar deriviatives. There are at least three known enzymes of this type: (i) a- amylase (ii) b-amylase (iii) starch phosphorylase.

Test for Starch: Starch gives a blue-black colour with dilute iodine solution.

CUTTING FREE HAND SECTIONS

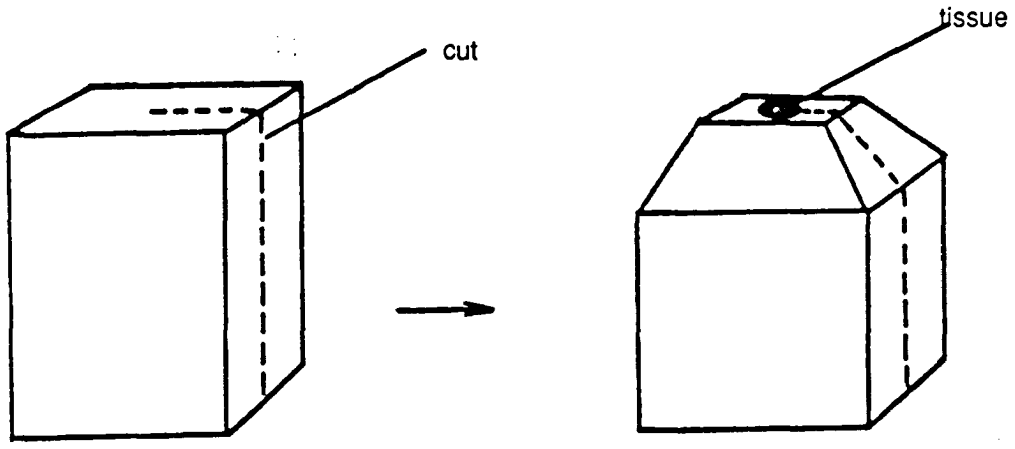
The cutting of the hand sections is the quickest and cheapest way of getting a pretty good idea of the anatomy of plants

A new razor blade of the thin, strong, single-sided type e.g. "valet" blades is recommended for cutting hand sectins.

Procedure for cutting sections

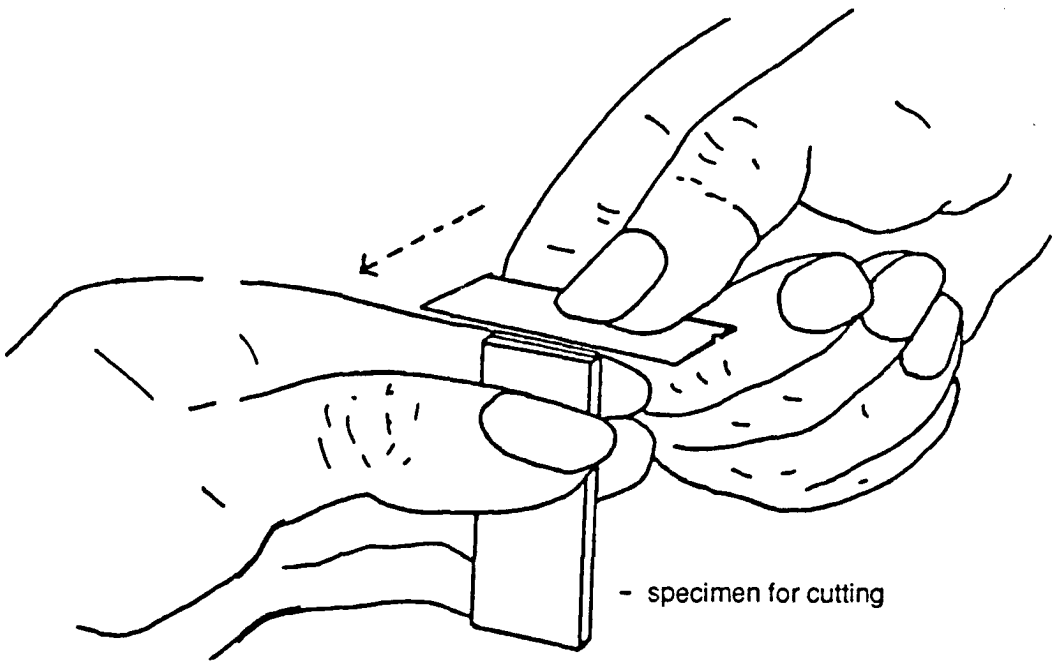
(1) Have ready some clean slides and cover slips, a butter-dish or watch-glass of water (if cutting fresh material) or 50% alcohol (if cutting preserved material).

(2) Place the piece of plant material in a slit piece of polystyrene or carrot. This step may be omitted for rigid plant material such as stems.Trim the block to eliminate excess polystyrene around the tissue. A blunt razor blade can be used for this. Reserve a new blade for the sectioning.



(3) Hold the block or piece of tissue between thumb and forefinger of the left hand with the thumb a safe distance below the surface.

(4) Flood the razor blade with water or alcohol and with the left forefinger supporting the razor draw it horizontally across the specimen towards you. Support both hands on the bench while cutting.



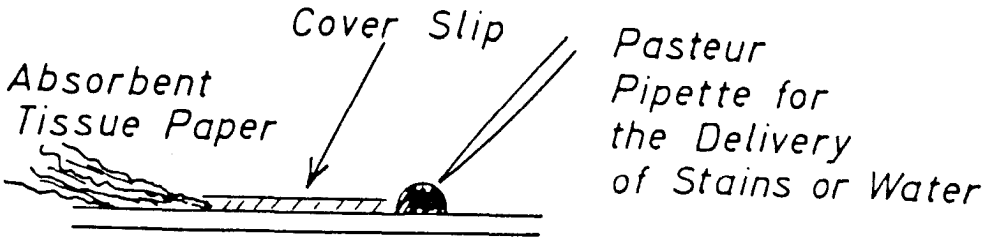
(5) Cut about 10 sections (it does not matter if sections are incomplete) in this way and then transfer them with a brush from your razor to the watch-glass or liquid. Always keep the specimen and the blade wet while sectioning.

(6) Select your best sections for examination or staining. Sections of fresh leaves are best examined mounted in water. Other sections are best stained and mounted in dilute glycerine (30 to 50% by volume).

NOTE: Many plant tissues contain air in their intercellular spaces. Such air pockets interfere with the image under the light microscope but they can be removed quite easily by placing the sections in a glass vial, half filled with water, and evacuating until no more air bubbles escape from the tissue.

Anatomical and cytological details can be seen more clearly if appropriate stains are used. A number of stains will be available and you will have to experiment with all of them before you achieve acceptable results with each particular tissue.

Place the sections on a microscope slide and a coverslip on top. Stain by subirrigation while you monitor the progress of staining under a light microscope. After the sections have stained to your satisfaction, wash off excess stain by subirrigating the preparation with water.



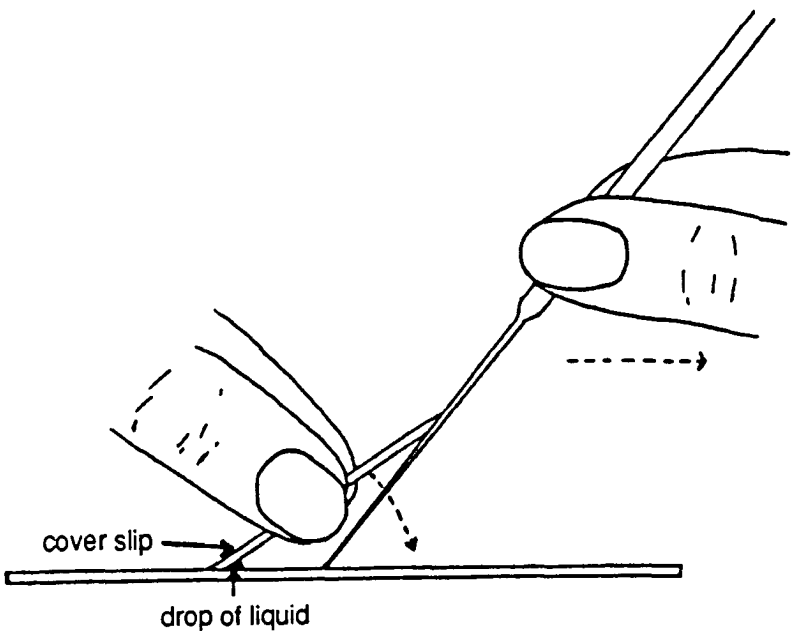
Fresh sections, mounted on slides, can be maintained for a few days if mounted in glycerol rather than water or sealing the edges of the coverslip with vaseline to prevent evaporation of the water. Drying of the preparations can also be prevented by keeping unsealed slides in petri dishes in which a piece of water-saturated filter paper provides a humid atmosphere. Sealed or unsealed preparations will keep better if placed in a refrigerator.

Note, however, that some tissues may be too delicate to survive these treatments.

MACERATED MATERIAL

Plant tissues may be separated into their component cells by removing the substances present in the middle lamella which "cement" the cells together (mainly calcium pectate). Thin slices of the material are macerated in a mixture of equal parts of aqueous nitric acid (10%) and aqueous chromic acid (10%). If the tissue is woody, heat is applied. Once the cells have separated, the acids are removed by washing in water. The macerated material is stored in 50% alcohol which contains safranin (1% by volume). A small portion of the macerate may be removed and mounted in dilute glycerine and examined. Macerated material is especially good for examining the lignification on the xylem vessels and useful for measuring the size of the various cell types.

MOUNTING SECTIONS



1. Wet Mount

Sections for examination are usually mounted in water or dilute glycerine. When examining a slide for a long period dilute glycerine is preferred as it does not dry up quickly.

(a) Place a drop of the mounting medium (water or dilute glycerine) in the centre of the clean slide lying on a clean level surface.

(b) Hold the coverslip by the edge between the left thumb and forefinger and place the coverslip at an acute angle to the slide with the edge of the coverslip just touching the drop of mounting medium.

(c) Once the mounting medium has spread along the edge of the cover slip, support the coverslip with a needle (held by the other hand) placing the point of the needle on the slide. Gently lower the cover slip by tilting the needle. Use a tissue to clean excess mounting medium from the slide.

2. Semi-permanent mounts

Wet mounts will dry out quickly. If the preparation is to last for a couple of days, after mounting the sections in dilute glycerine, clean excess glycerine and then seal the edge of the cover slip with nail varnish.

If a more permanent mount is required, mount sections in glycerine jelly. This method is particularly useful when making whole mounts (e.g. fern prothallus) and will last for several years.

Formula for Glycerine jelly

Gelatin	1 part (by weight)
Glycerin	7 parts
water	6 parts

Warm gelatine in water for 2 hours; add glycerine and warm, stirring, for 15 minutes. Add 1% phenol as a preservative. Store in airtight jars in the dark.

Procedure for semi-permanent mounts

(a) Remove the stained sections from the wet mount and place in a butter-dish containing dilute glycerine.

(b) Remove a small quantity of glycerine jelly from the jar (the size of a match head) and place on a clean slide.

(c) Place the slide on a hot plate with a temperature just warm to the touch. Let the glycerine jelly just melt (DO NOT LEAVE THE SLIDE ON THE HOT PLATE FOR LONG TO AVOID BOILING OF THE GLYCERINE JELLY WHICH WILL LEAD TO THE FORMATION OF AIR BUBBLES). Remove from the plate and place on a clean level surface.

(d) Remove the section/sections from the butter-dish with a fine brush and place on the melted glycerine jelly. Place a clean cover slip on the slide following direction for mounting wet mounts (previous page).

(e) Leave the glycerine jelly to set, trim off excess jelly and clean slide. Seal the edge of the cover slip with nail varnish and allow to dry. Label slides (Name of specimen, type of section, stain used, your name, date). The slides should be stored flat and in the dark.

SOME SIMPLE STAINING TECHNIQUES USED IN THE STUDY OF PLANT ANATOMY

The following simple staining methods are commonly used for making temporary mounts.

1. **IKI** (Iodine dissolved in alcoholic potassium iodide). This is a useful general mountant. Place sections in a drop of iodine solution for 5 minutes; drain off excess iodine, and mount in dilute glycerine. Alternatively, sections can be examined directly in the iodine solution. The stain fades after an hour or so.

   Cellulose walls are stained yellow
   lignified and suberised walls are stained brown
   starch is stained blue/black
   protein is stained brown

2. **Toluidine blue.** Stain with toluidine blue for 1 to 2 minutes, wash in water for one minute (to prevent over-staining) and mount in dilute glycerine.

   lignified walls are stained blue
   cellulose walls are stained purplish

   Use 2 staining dishes. put stain in one, water in the other. Transfer section from stain to water with a paint brush.

3. **Aniline double stain.** This is an aqueous solution of a mixture of aniline chloride and aniline blue. It is a useful stain for woody tissues.

   Place a section in a drop of solution for several minutes, drain off and mount in acid glycerine.

   lignified walls are stained yellow
   cellulose walls are stained blue

4. **Phloroglucin + HCl.** This is a 5% solution of phloroglucin in alcohol, to which strong hydrochloric acid is added. It is a useful stain for woody tissues but the acid vapour will etch microscope objectives if used consistently.

   Place a section in a drop of stain for several minutes, drain off and mount in acid glycerine.

   lignified walls are stained bright red.

   (If colour is not given at once add more acid or apply heat but do not overheat as dye tends to crystallize out).

5. **Sudan III.** This contains 2% dye dissolved in 70% ethyl alcohol. It stains fat droplets (rather slowly, allow several minutes) red.

CARE:

1. If water or reagents are spilt on the microscope stage or elsewhere, they must be wiped off immediately.

2. Objectives, eyepieces and lenses must be kept clean. All cleaning should be done with special lens tissue or a soft clean tissue.

3. Always use a cover-slip when objects are being examined under high power.

PREPARATION OF TISSUES FOR LIGHT MICROSCOPY USING A MICROTOME

The procedures that are described here are fairly basic and have been selected for their general applicability. Many modifications and elaborations will be found in the literature, particularly in the application of various staining techniques, that have been devised for specific purposes.

The preparation of biological materials for the light microscope involves a number of distinct steps: **Fixation, Dehydration, Embedding, Microtomy** and **Staining.** It is a rather "unforgiving" sequence of operations in the sense that any mistakes committed at any stage cannot be corrected. If the fixation has been messed up ..... forget the rest! The problem, of course, is that normally one may not know this until after the final product of the long sequence of time consuming operations has been examined under the microscope.

FIXATION

The aim in fixing plant material is to kill the cells rapidly and in such a way as to keep them in as near to the living state as possible. The fixative commonly used contains alcohol which helps to harden the soft tissue, making the cutting of sections easier. Material to be fixed must be cut into small pieces to allow the fixative to penetrate. Common fixatives used for plant anatomical work are F.A.A. (formalin/acetic acid/alcohol) and F.P.A. (formalin/propionic acid/alcohol).

Formulae

	F.A.A.	**F.P.A.**
60% ethyl alcohol	90 ml	90 ml
glacial acetic acid	5 ml	-
propionic acid	-	5 ml
formalin (40% formaldehyde)	5 ml	5 ml

Since formalin is a toxic chemical, after fixing for 2-5 days, (depending on the size of the material), the fixative is removed and replaced by 50-70% alcohol for storage. Storage jars should be labelled on both the outside and inside.

Generally, fixation may be done as follows:

(a) Half fill a vial with the chosen fixative. Include in the vial a small label that identifies the specimen. Use pencil, or better still, India ink. Do not use biro because it will wash off.

(b) Place the plant material into a petri dish that contains solidified paraffin and with a **NEW** razor blade slice it into pieces, not more than 2-3 mm thick. The smaller the piece the better the fixation! This is particularly important if you are interested in the preservation of cytoplasmic features. Often fixation may be improved by slicing the tissue while it is partly submerged in a few drops of the fixative held in a depression of the paraffin in the dish.

(c) In most cases you will need to take care of the precise orientation of the cuts you make so that the subsequent orientation of the tissue blocks in the microtome will be made easy. Random chopping of the material is bound to result in preparations that will be difficult to orient and impossible to interpret. **So! Before you chop ..... think!** Decide on the final orientation of the sections you intend to get (longitudinal, cross, tangential, paradermal) and cut the original tissue accordingly.

(d) Carefully transfer the material from the petri dish to the vial with the fixative. If the tissue pieces float, remove the air from the intercellular spaces by aspirating with a water pump or a syringe.

DEHYDRATION

1. Wash the fixative off with three (3) changes of distilled water. Allow at least 10 minutes to elapse between changes. If fixation has been very long it will be necessary to prolong washing.

2. Dehydrate the tissue by passing through the following series of ascending concentrations of alcohols. The indicated times should be used as a guideline, they are not absolute. Bulky tissues will require longer times at each step.

Solution	Mixture	Time
A	10% Ethanol + 90% Water	15 min
B	25% Ethanol + 75% Water	15 min
C	40% Ethanol + 10% Tertiary Butyl Alcohol (TBA) + 50% Water	15 min
D	50% Ethanol + 25% TBA + 25% Water If you cannot complete the dehydration down to solution I, you may leave the tissues in solution D overnight or over the weekend.	15 min
E	50% Ethanol + 40% TBA + 10% Water	30 min
F	45% Ethanol + 50% TBA + 5% Water	30 min
G	25% Absolute Ethanol + 75% TBA + eosin or erythrosin*	30 min
H	100% TBA 3 changes,	30 min each
I	Equal parts of paraffin oil and TBA	60 min to overnight

*Eosin or erythrosin are used to stain the tissue so that it can be visible after embedding in paraplast.

INFILTRATION

1. Half fill a large specimen vial with melted paraplast (embedding medium) and allow to start solidifying.

2. Place the dehydrated material on top of the partly solidified paraplast, just cover with fresh paraffin oil/TBA mixture and place immediately in the vacuum embedding oven. The material will slowly sink through the melting paraplast. You may leave in oven overnight but not any longer.

3. After the paraplast has completely melted, pour off the liquid, replaced with molten paraplast and return the vial in the oven.

N.B. Used paraplast should not be discarded down the sink.

4. Repeat this change twice allowing 2-3 hours between changes. Some difficult to infiltrate materials will probably require longer infiltration times. Only trial and error will tell you!

N.B. Used vials can be reused for subsequent infiltrations after pouring off all the remaining paraplast. If necessary warm the vial to melt the remaining paraplast.

EMBEDDING

1. Pour some paraplast at the bottom of an embedding mould and before it solidifies carefully transfer the pieces of tissue into the mould and orient them (by using a dissecting needle warmed over a flame) as desired, bearing in mind the orientation of the sections you wish to obtain. Transfer and embed the label at the edge of the mould so that it can be visible when the paraffin block is removed from the mould.

2. Allow the paraplast to cool. The process may be accelerated by placing the moulds in cold water after first producing a "skin" on the surface of the block by blowing gently over the surface (if the "skin" is not formed and you submerge the moulds the paraplast will float away!).

3. Remove the paraplast block from the mould. It is possible to pop the block out of the mould without destroying the latter which can then be re-used.

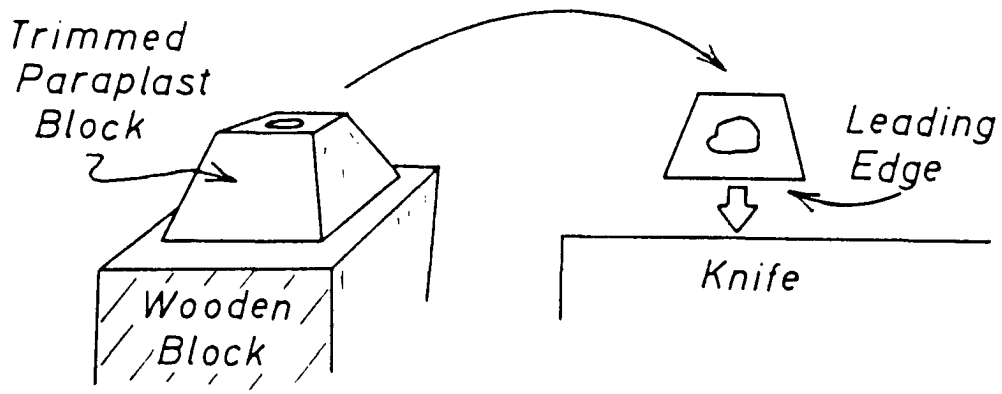
MICROTOMY

Preparation of the embedded tissues for sectioning.

1. Select and remove from the original embeddment a piece of tissue and mount it, properly oriented, on a wooden block.

2. Attach a copy of the original label to the wooden block.

3. Trim the paraplast (use a sharp razor blade) until you produce a truncated pyramid with only a small amount of paraplast around the tissue - make sure theat the leading and trialing edges are parallel.



Cutting of sections

1. Check that the advance mechanism of the microtome is fully to the rear, also that the knife carrier is at its furthermost from the block and that the locking device is engaged.

2. Place the wooden block in the microtome jaws and clamp securely. Carefully place the knife in the knife carrier, check the angle and clamp securely.

3. Move the knife up close to the block and clamp.

4. Carefully orient the block so that the three axes are correctly aligned with respect to the knife edge.

5. Release the locking device, set the cutting scale at 20μ and begin sectioning. When the first sliver of paraffin is cut, adjust the cutting scale to 10μ and resume cutting. You will probably have to experiment with various thicknesses before you achieve best results for your particular tissue. The recommended thickness of 10μ is a good starting point for plant materials.

6. As the sections come off the knife edge, grasp the end of the ribbon with a pair of forceps thus supporting the ribbon. When sufficient sections have been cut, remove the ribbon from the knife edge with a camel-hair brush (fine paint brush) and place it carefully on a clean piece of black paper where it will be easily visible.

Faults in sectioning

1. Ribbons are curved

   (a) Poor trimming, leading and trailing edge of block are not parallel
   (b) part of knife edge may be dull

2. Sections fail to ribbon

   (a) use low M.P. (Melting Point) paraffin
   (b) sections too thick or too thin
   (c) knife blunt
   (d) leading and trailing edge not parallel

3. Sections wrinkled or compressed

   (a) knife blunt
   (b) embed in harder paraffin
   (c) cool block in refrigerator
   (d) increase tilt of knife
   (e) reinfiltrate and re-embed

4. Ribbons scratched

   (a) nick in knife
   (b) grit or sand in specimen

5. Tissue crumbles of falls out of paraffin

   (a) poor infiltration, traces of water, alcohol or xylene
   (b) too long or too hot in paraffin
   (c) too long in xylene

6. Sections cling to block instead of knife

   (a) knife blunt or dirty
   (b) static electricity generated, breathe on block

7. Sections curl or adhere to parts of microtome

   (a) static electricity generated, breathe on block
   (b) knife blunt

8. Sections are missed and/or vary in thickness

   (a) loose part - check, particularly knife and block holder
   (b) sections too thin, increase thickness
   (c) knife tilt should be increased or decreased.

Mounting the ribbon on glass slides

1. Cut ribbons of appropriate length (usually 2-3 sections) with a rocking motion of the scalpel.

2. Clean a microscope slide thoroughly by wiping with paper towelling moistened with absolute ethanol.

3. Place the ribbon with the sections on the slide so that the "shiny" side of the ribbon is against the glass.

4. Carefully run the albumen solution (3 drops Mayer's Albumen in 15 ml distilled water) under the sections so that no bubbles are trapped underneath and heat gently in the spirit flame until the sections are expanded and flattened. **Do not melt the paraffin.**

5. Drain excess fluid from the slide, position the ribbon and wipe off remaining fluid with tissue paper. Make sure that you leave enough room at one end of the slide for a label. Label the slide with a glass marking pencil.

6. Place the slide in a rack and when all sections are mounted, place the rack in the incubator (40°C) and leave at least overnight. **This will ensure that the sections are firmly fixed onto the glass slide.**

STAINING OF SECTIONS

Before the sections can be stained, the embedding medium must be removed and then the slides should be brought gradually to a medium similar to that of the staining solution. Most stains are dissolved in water. The extent of hydration depends on the specific stains to be used. After staining, the sections are dehydrated through an ascending series of alcohol concentrations and finally mounted under a cover slip to make a permanent preparation.

The hydration schedule shown below is one of general applicability. All the solutions are kept in "coplin jars" that can hold anumber of slides simultaneously. Make sure that the side with the sections does not touch another slide; if this happens you are likely to damage your sections. Take care that in transferring a slide from one jar to the next you withdraw the slide slowly, and then blot the trailing edge before you place it into the next jar. This precaution ensures that he sections remain on the slide and that there is a minimal contamination of each subsequent solution by the previous one.

1.	Xylene* - Removal of paraplast	10 min
2.	Xylene - Completion of removal Make absolutely sure that all traces of paraplast have been removed. If necessary increase the time in Xylene.	10 min
3.	Xylene : Abs. Ethanol :: 1 ; 1	5 min
4.	Abs. Ethanol	5 min
5.	95% Ethanol	5 min
6.	75% Ethanol	5 min
7.	50% Ethanol	5 min
8.	25% Ethanol	5 min
9.	Dist. Water	5 min

*There are safer substitutes such as 'Histolene' on the market.

STAINING PROCEDURES

The staining schedules that follow are bound to produce some results but it is quite likely that you will have to experiment with specific steps to obtain best results for your materials. For example, the washing or destaining time indicated may be too long or too short for a particular tissue.

In the staining schedules that follow, the extent to which the sections on the slides must be hydrated is indicated in the first step.

Heidenhain's Iron Hematoxylin/Orange G

1. Remove paraplast and bring slides to water.

2. Transfer slides to mordant solution
   2% FERRIC AMMONIUM SULFATE for 1/2 - 2 h.

3. Wash gently and thoroughly in running tap water for at least 15 min.

4. Rinse in a change of distilled water. It is important to remove all traces of salts present in hard water.

5. Stain in 0.5% HEMATOXYLIN (aqueous solution ripened for at least 2 weeks) for 1/2 - 4 h; however some tissues may require longer staining times.

6. Wash off excess stain in running tap water followed by a rinse in distilled water.

7. At this stage the sections will be overstained and they must be destained and differentiated in 3% FERRIC AMMONIUM SULFATE until the various structures in the tissue appear to best advantage.

   CAUTION: Do not use the same coplin jar with the 3% FERRIC AMMONIUM SULFATE as in step 2.

The progress of destaining must be followed under a microscope. Remove the slide from the destaining solution, place it section side up onto a glass plate to protect the microscope from the liquids. Examine quickly to judge the progress of differentiation. Watch particularly for clarity of chromatin containing bodies. Take into account that the preparation will appear more grey in water than in the final mounting medium (due to differences in the refractive index). Destain until chromatin bodies look greyish-black.

8. Wash thoroughly in running tap water for 1 hour. If traces of the destaining solution remain in the tissues their continuous action will cause the preparation to fade completely.

9. Dehydrate the sections by passing the slides through an ascending series of ethanol: 25%, 50%, 75%, 95% and abs. ethanol. Do not leave for more than a few seconds in each step because the stain may be washed off with long exposures to alcohol.

10. Counterstain for 10-5 min in ORANGE G solution (0.5% in CLOVE OIL).

N.B. The use of a counterstain with Heidenhain's iron hematoxylin is not always necessary or desirable. Experiment with your materials and decide what is best!

11. Differentiate in : equal parts of CLOVE OIL: XYLENE; ABS. ETHANOL for 5-15 min. Monitor differentiation under microscope.

12. Xylene, 3 changes, 5 min each.

13. Mount coverslip with Depex or Euparal (synthetic resins)

The end product should not be sharply contrasting but show a gradation of shades from light grey to black.. This stain is particularly useful for the study of meristematic regions and other types of cells with dense cytoplasm.

Safranin - Fast Green

1. Remove paraplast and hydrate sections to 50% Ethanol.

2. Stain in SAFRANIN. Dilute stock solution of safranin (1% in 95% Ethanol) with an equal volume of dist. water before use. Stain for 1-24 hours, depending on the tissue.

3. Wash in water.

4. Pass briefly through 70% Ethanol.

5. Pass rapidly (a few seconds in each) through 95% Ethanol and then abs. Ethanol.

6. Counter stain with FAST GREEN (0.5% Fast Green in clove oil : abs. Ethanol :: 1:1) for 5-15 seconds. Hold the slide horizontally and flood the sections by delivering the appropriate volume of the stain with the pipette from the dropping bottle. **Do not discard the stain but pour it back into the dropping bottle.**

7. Differentiate the fast green by placing the slides in a mixture of CLOVE OIL: ABS. ETHANOL : XYLENE :: 2:1:1. Use two changes about 2 min each.

8. Pass the slides through 3 changes of xylene, at least 15 min each.

9. Mount coverslip with Depex or Euparal.

The saffranin will stain nucleoli, chromosomes, cuticle and lignified walls a brilliant red while the fast green should stain all other structures (cytoplasm and non-lignified walls) a bright green.

Johansen's Methyl Violet - Erythrosin

1. Remove paraplast and bring slides to water through hydration series.
2. Stain in 1% aqueous METHYL VIOLET 2B for 15-30 min.
3. Rinse off excess stain in water.
4. Simultaneously differentiate and dehydrate in a saturated solution of PICRIC ACID in 95% Ethanol. 10-15 seconds should be enough.
5. Stop the action of the acid by placing slides in alkaline 95% Ethanol (add 2-3 drops of ammonia/100 ml of 95% Ethanol) 14 seconds.
6. Wash in pure 95% Ethanol 15 seconds.
7. Counterstain in a saturated solution of ERYTHROSIN in ABS. ETHANOL : CLOVE OIL :: 1:1 for 5-10 seconds.
8. Clear in CLOVE OIL 30 seconds.
9. Pass through two changes of XYLENE.
10. Mount coverslip with Depex or Euparal.

This is a good stain for the demonstraton of **mitoses in meristematic** tissues. Resting and dividing nuclei stain a brilliant purple, cell walls red and cytoplasm pink.

APPENDIX 18

SUPPLIERS OF TISSUE CULTURE REQUIREMENTS

The following list provides the names of suppliers of chemicals, apparatus and equipment that are used in plant tissue culture.

GENERAL LABORATORY CHEMICALS

Selby Scientific and Medical
2 Kilroe Street, Milton, Qld
(07) 371 1566

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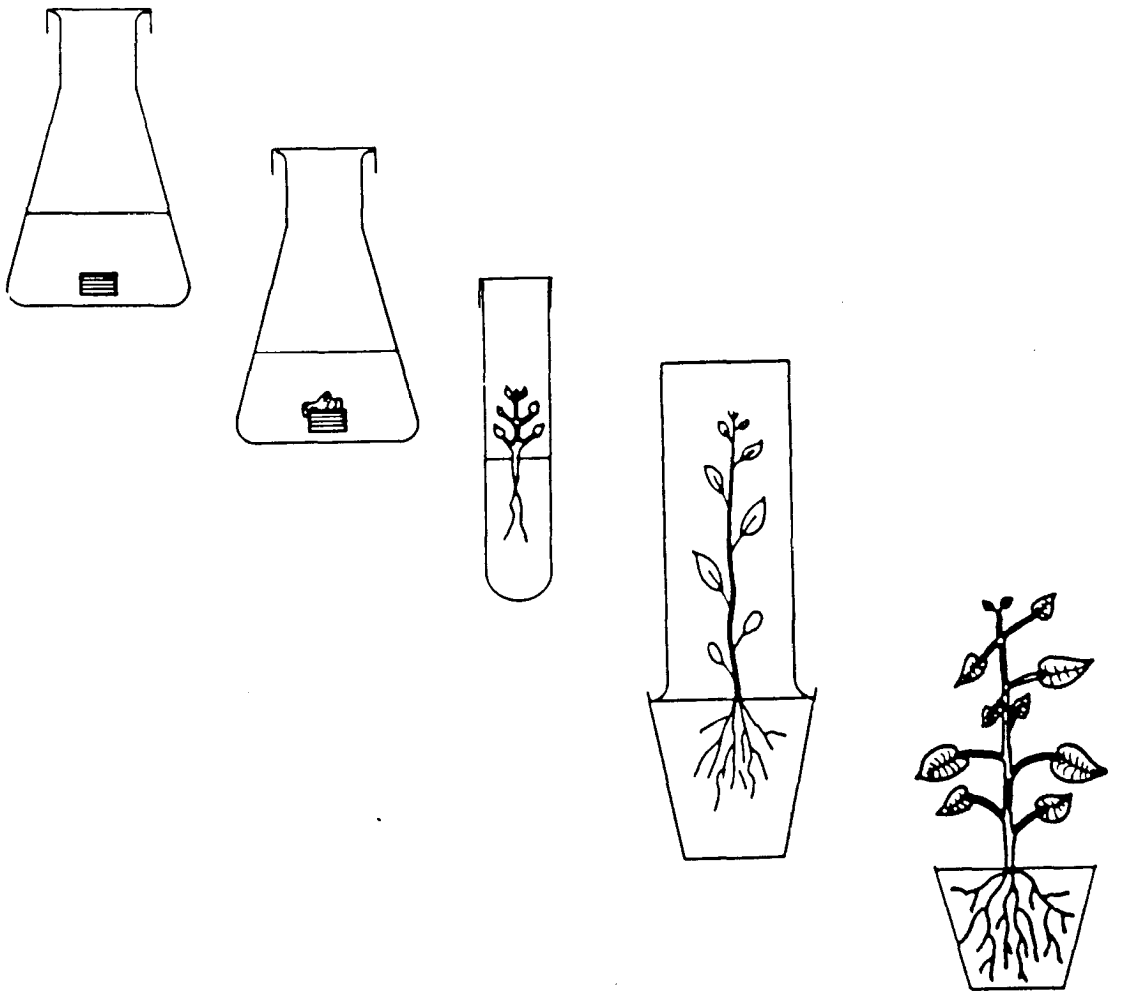

A GLOSSARY OF TISSUE CULTURE TERMS

Adventitious	An adjective describing the development of structures from abnormal positions, e.g. shoots from roots, leaves or callus.
Aerobic	Living in or dependent upon the presence of oxygen.
Anaerobic	Living in or dependent upon the absence of oxygen.
Aneuploid	Having fewer or more chromosomes than an exact multiple of the haploid number.
Anther	The part of the stamen which contains pollen in pollen sacs.
Anther culture	Tissue culture of anthers usually to obtain haploid plants.
Apical dominance	The phenomenon of suppression of growth of an axillary bud by the presence of the terminal bud on the branch.
Apical meristem	Meristem located at the apex of main shoot or lateral shoots.
Asepsis	Without infection or contaminating micro organisms.
Aseptic culture	The culture of a tissue or an organ in a medium free of bacteria, fungi, and other micro-organisms.
Asexual propagation	Propagation using vegetative parts of the plant.
Asynchronous	Not synchronous - see synchronous.
Autotroph	An organism that synthesises its own food from inorganic molecules, e.g. a photosynthetic organism.
Auxins	Hormones which cause cell elongation, apical dominance, root initiation. e.g. Indoleacetic acid (IAA).
Axenic	Uncontaminated or free of foreign organisms.
Axillary	Developing in the axil of leaves, e.g., axillary bud.
Basal Medium	A medium containing inorganic and organic nutrients but no additives such as growth regulators.
Backbulb	An older vegetative structure on sympodial orchids which may be leafless. It bears buds which can be stimulated to grow when the backbulb is separate.
Callus	A tissue arising from disorganised proliferation of cells - a group of undifferentiated plant cells.
Chimera	An organism comprising tissue of two different genotypes.

Chromosome	One of a set of thread-like structures found in the nucleus, which are composed of nucleic acids and protein. Chromosomes carry genes and are involved in the transmission of hereditary characteristics.
Chemically defined medium	A nutritive solution for culturing cells of known chemical composition.
Clonal Propagation	Asexual plant propagation starting from a single individual.
Clone	A population of plants derived from a single individual through vegetative propagation. More accurately it is a population of cells derived from a single cell by mitotic divisions. A clone is not necessarily homogeneous.
Coconut milk	Liquid endosperm of the coconut.
Continuous culture	A suspension culture continuously supplied with nutrients by the inflow of fresh medium with or without removal of biproducts.
Culture	Growing cells, tissues, plant organs, or whole plants in nutrient medium, under aseptic conditions, e.g. cell culture, embryo culture, shoot-tip culture, anther culture, etc.
Cybrid	A cytoplasmic hybrid, originating from the fusion of a cytoplast (the cytoplasm without the nucleus) with a whole cell.
Cytokinins	Growth hormones which cause cell division, cell differentiation, shoot differentiation, breaking apical dominance, etc. The cytokinin BAP is commonly used in tissue culture.
Cytoplasm	The parts of the cell inside the cell wall but not including the nucleus.
Deoxyribonucleic Acid (DNA)	The genetic material in every cell.
Differentiation	A term used to describe the formation of different cell types, roots, shoots, embryos, or any other organ in the callus or cell culture.
Dihaploid	This is an individual (denoted by $2_n = 2_x$) which arises from a tetraploid ($2_n = 4_x$).
Diploid	Having two sets of each chromosome characteristic for the species.
Disease-free	A plant certified through specific tests as being free of specified pathogens.

Disease-indexing	Disease-indexed plants have been assayed for the presence of known diseases according to standard testing procedures.
Disinfestation	Killing of micro organisms.
Electroporation	Creation, by means of an electrical current, of transient pores in the plasmalemma usually for the purpose of introducing exogenous material, especially DNA, from the medium.
Embryo	A very young plant developing inside the female gametophyte with or without fertilization.
Embryogenesis	The process of embryo initiation and development. See also somatic embryogenesis.
Embryoid	Non-zygotic embryo formed in culture.
Endogenous	Originating from within the organism.
Epigenetic variation	Non-hereditary variation which is at the same time reversible; often the result of a changed gene expression.
Excise	To remove a piece of tissue or an organ from its parent structure, e.g. separating individual shoots from a proliferating mass of shoots.
Exogenous	Originating outside of the organism.
Explant	A plant organ or piece of tissue used to initiate a culture.
Filter sterilization	Process of sterilizing a liquid by passage through a filter, with pores so small that they are impervious to micro organisms.
Gamete	A sex cell derived from a gametophyte and containing one-half the number of chromosomes present in the somatic cells of the plant.
Gametophyte	The haploid phase in alternation of plant generation which forms gametes.
Gene	One of a set of units of heredity, having a specific effect on the characteristics of an organism. Genes are composed of DNA (deoxyribonucleic acid) and are arranged linearly on the chromosomes.
Genome	The genetic endorsement or complement of an organism or individual.
Genotype	The genetic make-up of an individual as determined by the set of genes carried on the chromosomes.

Habituation	Ability of cells to grow in the absence of phytohormones after prolonged cultivation in the presence of hormones.
Haploid	Having a single copy per cell of each chromosome characteristic of the species.
Hardening off	Gradual acclimatization of in vitro grown plants to in vivo conditions, e.g. gradual decrease in humidity.
Heterokaryon	A cell in which two or more nuclei of unlike genetic make-up are present.
Heterotroph	An organism which is unable to carry out photosynthesis and thus cannot synthesise carbon compounds from simple inorganic molecules. Such carbon compounds (e.g. sugars) which are used as a source of energy and units for synthetic reactions must be obtained from the environment in which the heterotroph lives.
Heterozygous	Having different alleles at one or more corresponding loci or point in homologous chromosomes. Self-fertilization of heterozygous individuals would give a heterogeneous population.
Homozygous	Diploid or polyploid individuals having identical alleles on the homologous chromosomes. Self-fertilization of homozygous individuals would give a homogeneous population.
Hybrid	An organism or cell line resulting from a cross between parents that are genetically unlike.
Hybridization	Any process by which hybrids are created.
In vitro	Literally "in glass", now applied to any process carried out in sterile cultures.
In vivo	Literally "in life", applied to any process occurring in a whole organism.
Induction	To cause initiation of a process or a structure.
Inoculate	Place inoculum in or on a nutrient medium.
Internode	The portion of stem between two nodes.
Juvenility	Describes the early stage of growth in which the plant is in a rapid vegetative growth phase.
Karyotype	The characteristic number, size, and shape of the chromosomes in the metaphase of a somatic cell.
Mericlone	An orchid clone, originating from a meristem or other organs isolated in vitro.

Meristem	A localised group of actively dividing cells, from which permanent tissues (e.g. root, shoot, leaf, flower) are derived.
Meristematic	Having the characteristics of a meristem.
Meristemoid	A localized group of meristematic cells that arise in callus tissue and may give rise to roots and/or shoots.
Micropropagation	Asexual or vegetative propagation of plants in vitro.
Microspore	A uninucleate, haploid cell which develops into a pollen grain (male gametophyte).
Morphogenesis	The development of form or structure.
Mutagenesis	A process which changes the genetic constitution of a cell through alterations in its DNA.
Mutation	A change in the amount or structure of DNA in an organism, resulting in a change in that organism's phenotype.
Mutant	An individual having a different genotype from the parents and a different phenotype.
Nitrogen fixation	The conversion of atmospheric nitrogen (N_2) into ammonia and amino acids.
Node	A region on the stem from where a leaf is attached, usually bearing an axillary bud.
Nutrient medium	A solid or liquid combination of inorganic and organic nutrients and water.
Off-type	A plant produced from tissue culture which exhibits phenotypic variation, i.e. is a mutant.
Organ culture	Aseptic culture of organised structures, e.g. root tip, shoot tip, shoot segments, embryo, etc.
Organogenesis	The formation of organs.
Parenchymatous	Adjective used to describe undifferentiated cells with primary cell walls, capable of both cell division and differentiation.
Passage	The routine transfer of cells or tissues to new medium.
Pathogen-free	Plant, meristem, tissue, or cell, which is free of diseases (bacteria, fungi, viruses, etc.).
Phenotype	The characteristics of an organism due to the interaction of genotype and environment.

Plantlet	A small rooted shoot or germinated embryo.
Ploidy	The number of full chromosome sets per nucleus.
Pollen	Older microspore. Mature pollen bears the male gametes (sperms). Upon reaching the right stigma (tip of the female sex organ) the pollen germinates, with the production of a pollen tube carrying the male gametes to the egg.
Polyembryony	Development of more than one embryo in a seed, or multiple embryo development in cultures.
Polyploidy	Containing three or more sets of chromosomes per nucleus (triploid, tetraploid, pentaploid, etc.).
Primordium	Group of cells which give rise to an organ.
Propagule	A small piece of a plant used in propagation.
Protocorm	In orchids, seeds contain an unorganised embryo comprising only a few hundred cells. During seed germination the embryo first forms a tuberous structure called a protocorm, from which develops a complete plant. The vegetative parts of some orchids in culture form round, glistening, protocorm-like structures which may be multiplied indefinitely or induced to regenerate a whole plant.
Protoplasts	Single cells from which the walls have been removed.
Solid media	Nutrient media solidified, e.g. with agar.
Somaclonal Variation	Genetic variation which occurs in plant cells during culture. The source of variation is unknown.
Somatic	Referring to vegetative or non-sexual part or process.
Somatic embryogenesis	Embryoids arising from somatic cells: no sexual process is involved.
Sporophyte	The diploid phase in plants with alternation of generation which produce spores.
Subculture	Sub-division of a culture for transfer to fresh medium.
Suspension culture	Cells or cell aggregates cultured in liquid medium.
Sympodial	Plant development where the terminal bud of the stem ceases growth, often after flowering and the uppermost lateral bud then assumes the role of the terminal bud.
Synchronous culture	A culture in which most of the cells undergo cell division at the same time. Opposite to asynchronous.

Tissue culture	A general term referring to all types of aseptic plant or animal cultures.
Totipotency	The property of a cell to develop, divide and differentiate into an entire organism. Totipotent cells contain all the genetic information for complete development.
Transfer	The process of relocating cultured tissue to fresh medium. Alternatively called passage.
Vegetative propagation	The asexual propagation of plants by detachment of some part of the plant body, e.g. a cutting, and its subsequent development into a complete plant.
Virus-free	A plant certified through specified tests as being free of specified viruses.
Vitrification	Describes unthrifty cultures which appear waterlogged, translucent or glassy.
Zygote	The cell formed by union of two gametes (sperm and egg cell).

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