Plant Tissue Culture as a Source of Biochemicals

Editor

E. John Staba, Ph.D.

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PREFACE

Plant tissue culture techniques are profitably used today throughout the world. The technique is used to rapidly and uniformly propagate many horticultural plants, occassionally important food plants — for example, potato and sugar cane, and economically valuable plants containing steroids and pyrethrins. Academic, government, and industrial laboratories are also developing tissue culture techniques to rapidly propagate trees; to develop plants which will resist specific pathogens or will grow better in unfavorable environments; to be used for bio-assays; or to introduce desirable nuclear- or epi-genetic factors into plants. Aseptic plant cells, tissues, and organs are also used to better understand the biochemical processes of a plant. Although some microbial procedures are applied to plant tissue culture systems, plant cells are dramatically larger, and genetically and physiologically more complex than microorganisms. The major objectives of this book are to inform the reader of these significant differences, and of the scientific progress made in growing aseptic plant cell suspension cultures for biochemical production.

After approximately two decades of study it has been demonstrated that serially propagated plant tissue culture systems will produce many unusual secondary compounds; that cell strains can be selected for high production of some alkaloids and pigments; that some plant tissue cultures can be stored for long periods; and that plant cells can be grown in fermentors containing thousands of gallons of medium. However, several problems remain to be solved. For example, some compounds of interest are not produced in detectable or adequate amounts; selected strains are not always stable; and growth may be undesirably long and costly. Regardless of these important considerations, plant biochemical systems are *now* available for use in a controlled environment of our choosing. It should be remembered that single plant cells are totipotent in that they can be regenerated into whole plants. These cells are probably also biochemically equivalent to the plant at selected stages of cell, tissue, organ, or plant growth. Today, the art of plant tissue culture has become a science that is very much in an active developmental phase.

Although the obvious use of plant tissue culture systems to produce biochemicals industrially is not yet realized, one should not ignore the advantages of being able to produce plant substances independently of weather, geography, or even the confines of the surface of the earth. Indeed, the use of plant tissue culture may in the foreseeable future become greatest in space colonies!

I wish to thank the many contributors to this book, some of whom are easily identifiable as they have authored chapters. Others are not as easily identified to the reader, as they contributed by their correspondence and their personal suggestions to the authors. Lastly, I wish to thank some of the dedicated scientists to this research specialty whose works continue to inspire us — G. Morel, H. E. Street, and P. R. White.

E. John Staba

THE EDITOR

E. John Staba, Ph.D., Professor of Pharmacognosy, College of Pharmacy, University of Minnesota, was born May 16, 1928 in New York City, New York. He received his B.S. (Pharm.) (1952) from St. John's University; M. S. (1954) from Duquesne University; and Ph.D. (1957) from the University of Connecticut. He is married to Joyce E. Staba and has five children, three girls and two boys. He was Assistant Professor of Pharmacognosy (1957) and Professor and Chairman of the Pharmacognosy Department (1965) at the University of Nebraska. In 1968 he assumed the position of Professor and Chairman, Department of Pharmacognosy at the University of Minnesota.

He served as a consultant for the U.S. Army Quartermaster Corp., National Aeronautics and Space Administration (NASA) and various pharmaceutical and industrial concerns. His publications (60+) and patents (2) have been in the area of medicinal plant tissue culture, aquatic plant phytochemistry, and pharmacy education. He received the Lunsford-Richardson Honorary Research Award (1957), and was a Gustavus and Louise Pfeiffer Visiting Professor, University of Connecticut (1966). In 1969 he was a National Academy of Sciences Visiting Scientist to Poland, Czechoslovakia, and Hungary. In 1970 he was a recipient of a Fulbright Hays Research Fellowship to Germany, and in 1973 a Council of Scientific and Industrial Research (CSIR) Visiting Scientist appointment to India. In 1974 he was an invited participant in a U.S.-Republic of China Cooperative Science Program Seminar on Plant Cell Tissue and Cell Culture, and a Vice President and participant in the 3rd International Congress of Plant Tissue and Cell Culture, Leicester, England. He has participated as a member of the visitation team for the American Foundation for Pharmaceutical Education (1976); presided over the Pharmaceutical section of a special NASA Colloquium on Bioprocessing in Space—March, 1976; and was invited by the German Ministry of Science and Technology to participate in an International Congress on Plant Cell Cultures and their Biotechnological Applications (Munich, September, 1976). He is listed in Who's Who in America, and American Men of Science.

He has served on many committees of the Academy of Pharmaceutical Sciences (Section of Pharmacognosy and Natural Products), the American Society of Pharmacognosy, the American Association of College of Pharmacy, and the Tissue Culture Association. He was President of the American Society of Pharmacognosy (1971—72), Chairman of the AACP Conference of Teachers (1972—73), and Chairman of the Plant Division, Tissue Culture Association (1972—74). He was a member of the In-Vitro Editorial Board, Tissue Culture Association (1974—76).

He served as Vice Chairman (1975—76) and Chairman-elect (1976—77) of the Academy of Pharmaceutical Sciences Section on Pharmacognosy and Natural Products. He also served as the AACP Council of Sections representative to the AACP Board of Directors' (1976—78).

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Chapter 1

LABORATORY CULTURE

Janet E. A. Seabrook

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I. INTRODUCTION

Plant tissue culture is the technique of growing plant cells, tissues, and organs in a prepared nutrient medium and in the absence of microorganisms. There are two main applications of plant tissue culture. As a unique and valuable research tool, growing plant tissues in vitro can help minimize variables such as environmental factors. Better control can be attained of light, temperature, gas mixtures, and nutrition. Correlative influences, which are working relationships between two or more organs within a plant, can be reduced. Plant tissues can be grown in the absence of artifacts attributable to bacteria, fungi, algae, small organisms, and possibly, viruses. Plant tissue culture can also be employed to preserve valuable germ plasma and as a tool for the plant breeder.

A recent application of plant tissue culture is the use of this technique for the production of economically valuable chemicals. Plant cells and tissues in culture can be manipulated so that specific chemicals can be extracted from the cultured tissues or from the medium in which the tissues have grown. In addition, the principle of totipotency, which states that every cell within the plant has the potential to regenerate into a whole plant, can be applied to regenerate plants from cultured cells and tissues. The propagation of valuable economic plants through tissue culture is based on the principle of totipotency.

A general feature of plant tissue culture is the disinfestation of the tissue to be cultured. A suitable nutrient medium has to be selected and, then, appropriate environmental conditions chosen for the kind of tissue to be cultured and for the type of culture desired.

For both research and economic purposes, most plant tissue culture involves the growth of callus on a semisolid medium. Liquid-suspension cultures of cells are not as widespread, probably due to the cost of equipment. Commercial plant propagators use cultures started from shoot tips or lateral buds, sometimes erroneously referred to as "meri-cloning" or "meristem cultures." Plant pathologists culture meristems in an attempt to obtain virus-free plants. Because of the potential for possible dramatic strides in genetic modification and crop improvement, the fusion protoplasts have also become a popular field of research in recent years.

This chapter will attempt to consider the parameters associated with the establishment of callus from explants, factors required for the growth of callus in culture, and the subsequent regeneration of plants from callus. Appendix I provides a sample methodology for the induction of callus and for the regeneration of shoots.

The information we have at present on the culture of plant tissues in vitro may not be applicable to all plants. Techniques of regenerating plants by auxillary branching, adventitious shoots, etc. have been discussed in a recent review by Murashige[1] and will not be repeated here.

II. THE SOURCE PLANT

The successful production of callus and subsequent plant regeneration is dependent, in part, upon the qualities associated with the explants used. These, in turn, are related to the condition of the source plant.

Often, there is considerable variability associated with the genotype of the plants used as explants.[1] While it has been convenient to categorize success in vitro with taxonomic grouping, this classification has not been entirely valid. There is as much variability in vitro between cultivars of the same species as there is between families. The ease of organ regeneration, e.g., root regeneration, in vitro is usually related to the ease of vegetative propagation by traditional nursery methods. Conversely, plants which are difficult to propagate vegetatively are also often difficult to regenerate in vitro.

Tissue culture should normally be viewed as a situation that allows for the enhancement of a process which can be observed in nature, i.e., as a means to enhance an ability already present. Tissue culture is not the miracle which will allow for the expression of genetically nonexistent characters.

A. Organ Source

The tissue or organ used as a source of explants can also be a determinant of the success, or degree of success, of a plant tissue culture. It is advisable to compare the culture of various organs and tissues systematically for each plant before selecting any given tissue or organ.

Even though totipotentiality may be a universal characteristic of plant cells, its expression has been limited to certain types of cells, identified as meristemoids by Torrey.[2] Meristemoid cells are apparently cells that respond to organogenetic stimuli, such as auxin/cytokinin balances. They can be distinguished among cultured cells by their relatively small size, dense cytoplasm, isodiametric shape, thin cell walls, minimal vacuolation, and large nuclei. They usually occur as clusters within the cultured tissues and sometimes appear as nodules or proembryonic masses.

If whole-plant regeneration is desired, the presence of meristemoids, or cells which can readily develop into meristemoids, are necessary. Such cells have been found in shoot tips of *Asparagus*,[3] immature leaf bases of *Narcissus*,[4] *Osmunda* leaf tips,[5] *Pseudotsuge menziesii* cotyledons,[6] *Citrus* nucellus,[7] *Citrus* ovary,[8] *Daucus carota* root,[9] *Nicotiana* stem internode,[10] and *Datura* microspores.[11]

B. Developmental Phase

Juvenile plants or growths usually provide more regeneration explants than adult plants. The juvenile and adult phases are reversible to varying degrees, the ease of reversibility being dependent on the plant species. Both juvenile and adult growth can be found on the same plant at the same time. The juvenile phase is characterized by vigorous vegetative development and the absence of reproductive-structure formation. The adult material usually grows more slowly and is sexually reproductive. The two phases can sometimes be distinguished by different leaf shapes and other morphological characteristics.

C. Ontogenetic Age

Even among materials in the juvenile development phase, tissue and organ regeneration is more likely to be accomplished with the younger tissues. Young plants provide the best explants. Older plants may have accumulated more pathogens in their tissues and, therefore, not be as healthy. Furthermore, older plants probably contain less meristematic tissue and may have the additional problem of being physiologically mature. Unfortunately, one often does not know if plants have characteristics which are desirable (e.g., superior blooms, higher fruit yields, production of a biochemical) until they are physiologically mature. This is a particular problem with woody perennials. Murashige[12] has illustrated the relationship between ontogenetic age and the organogenetic behavior of tissues in vitro.

D. Preculture Treatment of the Source Plant

Preculture treatment of source plants can make the difference between the success and failure of a culture. Standard greenhouse conditions such as soil mixtures and fertilizer applications should be maintained with as much uniformity as possible.

1. Seasonal Requirements

The position of the explant on the source plant[12] and the season of the year can make a difference to the regeneration of *de novo*-formed bulbils on *Lillium* bulb scales.[13,14,15] Fellenberg[16] noted that organogenesis in *Solanum tuberosum* tissue cultures was seasonally mediated. Thus, climatic requirements such as photoperiod[17] and temperature (cold treatment) may have to be satisfied prior to culture.[15]

2. Nutritional Requirements

The nutritional status of the source plant probably affects the successful establishment of explants in tissue culture. Heavy applications of fertilizer to the source plants prior to their use as explants for tissue culture can drastically change the response of the explants to the cultural conditions.

3. Etiolation

Changes in the light intensity to which source plants are subjected can alter the response of tissues to experimental conditions.[18]

4. Pretreatment with Growth Regulators

Pretreatment of the source plants with growth regulators can enhance or change the response of the explants to cultural conditions. Pretreatment with gibberellic acid can, in some plants, produce juvenile tissue which is easier to culture.[19]

The application of the growth retardant CCC (β-chloroethyltrimethyl ammonium chloride) as a foliar spray on tomato plants before using them as a source of culture material altered the response of the resultant callus to the application of exogenous auxin and cytokinin.[20]

5. Reduction of Microorganisms by Pretreatment of Source Plant

Explants which are free of microorganisms, especially pathogens, can be obtained by pretreating the source plant with antibiotics or with fungicides. Treatment of source plants with antiviral agents such as "virozol", heat-treatment, and drying out can all reduce the microorganism content of the explant in culture.

Plants established in growth chambers usually provide the cleanest explants for culture, whereas green-house-grown and field-grown plants provide explants which are successively more difficult to rid of microorganisms.

E. Explant Size

The larger the explant, the greater the danger of the inclusion of microbial contaminants in the tissues. There is, however, a minimum effective size for explants, as very small explants such as meristems do not grow as fast or respond as effectively to cultural conditions as do larger explants. This dilemma can be partly solved by culturing several small explants together in one culture vessel. In this way, shock due to dissection and transfer are also reduced. There appears to be a "population effect" in vitro. When several small explants are cultured in the same vessel they grow faster than small explants cultured singly.

F. Disinfestation of Tissue to be Used for Culture

It is always necessary to remove all microorganisms from tissue to be cultured because they will outgrow the tissues and destroy them. Secondly, the presence of microorganisms changes the environment by removing nutrients from the medium and by releasing metabolic by-products into it. Results obtained from contaminated cultures may not be reproducible. Surface sterilization is particularly difficult with hairy or unevenly surfaced materials as they harbor pockets of microorganisms. Occasionally a combination of more than one kind of disinfesting agent is effective. A compromise has to be achieved between adequate disinfestation and causing as little damage as possible to the tissues to be cultured.

Plants to be used as a source of explants should be removed from the field or greenhouse and all soil washed from the roots. If only small sections of a large plant are required, the tissue can be removed with a sharp scalpel and the cut surface dipped in hot wax before transport to the laboratory. All necrotic, old, or obviously unclean tissue should be removed before applying sterilizing agents. Sharp scalpel blades should be used to prevent unnecessary damage to the explants. When suitable, outer tissues such as bud scales and older leaves can be left on until after surface-sterilization. They can then be removed under sterile conditions.

A mild soap and running tap water can be used to remove much of the surface dirt from tissue to be cultured. Placing the tissue in 70% ethylalcohol for 5 to 20 sec removes some contaminants. The most common method of surface-sterilizing plant material is the use of a chlorine solution, Purex® sodium hypochlorite, or calcium hypochlorite for 5 to 30 min (15 min is usually sufficient).

Penetration of the disinfesting agent into uneven or hairy surfaces can be promoted by the addition of a wetting agent such as Tween 20® (1 to 2 drops per 100 ml disinfestant, ca. 0.05%). Placing the explant and surface sterilant together in an erlenmeyer flask on a rotary shaker (50 to 150 rpm) for 15 to 30 min enhances disinfestation.[21] The use of a vacuum can further increase penetration of the disinfestant into the surface crevices of the tissue.

After disinfestation, all manipulations should be performed in a sterile environment. Utilizing a laminar-flow transfer hood is the most effective way to ensure sterility of material to be cultured. All equipment placed in a transfer hood should be sterile or

swabbed with alcohol or Purex®. Sterilized tissue should be rinsed at least three times with sterile, distilled water. This is best done in the transfer hood.

All damaged or dead material should be removed from the disinfested explant which is now ready to be planted on a suitable nutrient medium.

III. ESTABLISHING A CALLUS CULTURE

The most commonly cultured plant tissue is callus, which is wound tissue composed of differentiated, highly vacuolated, unorganized cells.

Callus and cell-suspension cultures of most plant species have been easier to culture than shoot cultures. Callus is usually obtained by culturing explants on a semisolid medium containing a high concentration of salts, high auxin, and casein hydrolysate. Once friable callus has been obtained, the tissue can be transferred to a liquid medium. If vigorous agitation and aeration is applied, a suspension of free cells and aggregates of cells can be obtained. Callus cultures are usually maintained in darkness, and 2,4-D is frequently added to suppress organogenesis.

A. Media Formulations

Although whole plants have simple requirements for growth, plant-tissue cultures have more complex needs and are seldom autotrophic. That is, plant tissue in vitro requires the usual macro- and microelements supplied in hydroponic culture. In addition, other nutrients, such as a source of bound carbon and vitamins, are necessary. Isolated plant cells and tissues frequently require the addition of vitamins and plant-growth regulators that in vivo are synthesized by one part or organ of a plant and transported to another part where they are metabolized. Little is known of the effect of many of the individual constituents on the production of secondary metabolites because the chemical composition of most plant-tissue-culture formulations have been devised with the aim of improving cell growth and organogenesis. Media composition for suspension cultures is discussed in Chapter 2. Thus, only generalities will be noted here.

1. Salt Requirement for Cultured Plant Tissues

Although early tissue-culture formulations[22,23] were suitable for the culture of callus cells, later formulations, such as those of Murashige and Skoog[24] and Gamborg, Miller and Ojima,[25] are more suitable for a wider range of plants and for the promotion of organogenesis in cultures. A formulation has to be determined which supports the growth of cultured cells for each species, and sometimes each variety within a species. Generally, the Murashige and Skoog (MS)[24] salt solution will support the growth of most plant cells in culture, and this is in part due to its high salt concentration. Some ions, the NH_4 ion in particular, may be in too high a concentration for all plants and tissue at all stages of development in vitro. The addition of $NaH_2PO_4 \cdot H_2O$ to the MS salt solution has been beneficial for some tissue. Huang and Murashige[26] have listed the major constituents of plant tissue culture media and have indicated how they can be prepared.

Even though there are trace impurities of minor elements in supplies of major elements such as N, K, and P, additional quantities of these microelements are almost always necessary. The MS salt solution is particularly high in microelements when compared to other media formulations.[26] The addition of chelating agents such as Fe-EDTA* ensures that iron is available over a wide range of pH.

* EDTA, ethylenediaminetetraacetic acid.

2. Osmolarity and Ion Concentration

Embryogenesis is more likely to occur when tissue is grown in a salt solution of high osmolarity such as the MS formulation.[27] The concentration of N and K ions appear to be important for somatic embryogenesis. Reinert[28] proposed an interaction between nitrogen and auxin which enhanced embryogenesis.

3. Organic Constituents

a. Vitamins

The use of vitamins in plant tissue culture media has been a matter of custom rather than proven necessity. Thiamine·HCl is the only vitamin for which there seems to be a consistent requirement for growth of plant tissues in vitro. Other vitamins should be added to media formulations where enhancement of growth or morphogenesis indicate they are necessary.[26]

b. Carbon Source

The addition of an organic carbon source, such as sucrose, to plant tissue culture media, is absolutely necessary for nearly all tissue, as very few plant cells in vitro are autotrophic. Sucrose, in concentrations of 2 to 3%, is the most commonly used carbon source. There is evidence that the production of some metabolites by plant tissue may be affected by the concentration of sucrose.[29]

c. Growth Regulators

Hormones, in intact plants, act to regulate and coordinate processes which lead to normal development. Growth, as well as differentiation of tissue and cells and secondary metabolism, is affected by these hormones. The addition of plant-growth regulators to tissue-culture media, is not always necessary for callus cultures. However, supplementation with growth regulators is usually obligate for callus cultures in which an increase in the growth rates or organogenesis is required.

Very few plant tissues in vitro will produce extensive callus in the absence of growth regulators.[30] *Helianthus tuberosus* callus from dormant tubers and callus from the root of *Cichorium* are autotrophic for cytokinin, but require auxin.[30]

Indole-3-acetic acid (IAA) is the most generally used auxin for plant tissue culture because it has fewer adverse effects on organogenesis than other auxins. IAA is weaker than naphthaleneacetic acid (NAA). NAA can sometimes be used in higher concentrations than IAA, which is usually used on concentrations of 10^{-5} to 10^{-10} *M*.[30] Media containing IAA deteriorates faster than media containing NAA, especially if not refrigerated.

The most potent of the commonly used auxins is 2,4-dichlorophenoxyacetic acid (2,4-D). It strongly suppresses organogenesis and is particularly useful for the maintenance of callus cultures. 2,4-D is generally active in plant tissue cultures at concentrations from 10^{-5} to 10^{-7} *M*.[30] It should be recalled that 2,4-D is a commonly used herbicide for the control of broadleaved weeds, and therefore, its use in cultures of dicotyledonous plants may be restricted.

A second class of commonly used growth regulators in tissue culture are the cytokinins. The most active of the common synthetic cytokinins is N_6-isopentenyladenine (2iP). N_6-Benzyladenine (BA) has a particularly enhancing effect on the release from apical dominance of axillary shoots and on the proliferation of shoots induced in vitro. Tobacco pith callus requires a concentration of 10^{-6} to 10^{-7} *M* kinetin for growth, whereas organogensis in tobacco pith callus needs a concentration of 10^{-5} to 10^{-7} *M* kinetin.[30] As there is some evidence that kinetin, which is a synthetic cytokinin, can be degraded by light (300 to 800 nm), care should be taken to store stock solutions in darkness.[31]

Skoog and Miller[32] reported that the balance of auxin to cytokinin levels had an effect on organ formation. Their concept states that both auxin and cytokinin are necessary for the control of growth and of organogenesis in vitro. The kind of development, i.e., callus, roots or shoots, is determined by the relative amounts of these two growth regulators. This concept should be applied when trying to obtain organ formation in cultures. Phenolic compounds[33] and excess gibberellins[20] can mask the effect of a balance of auxins to cytokinins and, therefore, suppress organ formation.

Gibberellins have been used in tissue culture, but they generally have the effect of suppressing organ formation.[10] The proporion of various growth regulators and the concentrations that are required for plant tissues varies with the stage of development of the cultured tissue.[1] Organ initiation is generally enhanced by higher levels of growth regulators than is required for the growth of callus.[1]

d. Amino Acids and Amides

The requirement for amino acids by a plant-tissue culture can be estimated by adding varying amounts of a protein hydrolysate (e.g., enzymatic casein hydrolysate). Any enhancement of growth or morphogenesis can be explored further by testing a mixture of amino acids and amides.[26] The use of amino acids in plant-tissue culture media should take into account possible antagonisms between them.

The amino acids and amides which commonly give beneficial effects are L-argenine, L-aspartic acid, L-asparagine, L-glutamic acid and L-glutamine.[26]

e. Nitrogen Bases

The nitrogen bases, cytidylic and guanylic acids, have been reported to enhance growth in callus cultures.[26]

f. Natural Complexes

Natural complexes, such as coconut milk, yeast extract, and protein hydrolysates (hydrolysates of casein, lactalbumin, peptone, and tryptone) which have been used in the past for plant tissue cultures, should be avoided. Quality control with these products is nearly impossible. Use can result in nonreproducible results.

A more effective way of supplementing media with organic constituents is to add various known chemicals when it has been determined that they improve growth or morphogenesis of cultures.

g. Materials which Enhance the Quality of the Medium by Physical Means

(1) Semisolid Media

In early plant tissue culture techniques, agar was used to solidify the medium. Only simple laboratory equipment is required for a semisolid medium, but the yields of biomedicals may not be as high as can be obtained from liquid cultures. Agar is still the most commonly used material for gelling media, however the quality should be very carefully monitored. Unless one is reasonably sure that the agar is pure, it should be washed at least three times with distilled water. Romberger and Tabor[34] found that the best growth of *Picea abies* was with a nutrient medium solidified with Difco®, "Purified" agar, and that Difco,® "Noble" agar, which is more refined, gave poorer growth. High concentrations of agar can inhibit the growth of plant tissue in culture. The precise concentration of agar suitable for each medium and plant tissue should be determined for each situation. However, a concentration of 0.8% is usually sufficient for most purposes.

Gelatin and silica gel have been used to support plant tissue, and recently acrylamide gels have been developed. Starch co-polymers have been suggested as agar substitutes.[35] They have the advantage that they do not require boiling to dissolve in

water. There are problems at present with adjusting the pH, and these will have to be solved before starch co-polymers can be widely used.

Charcoal is frequently added to media formulations. It enhances growth by adsorbing toxic metabolites of tissue cultures.[36]

(2) Liquid-Media Formulations

Liquid formulations are also common. Tissue can be continuously immersed in the liquid medium or suspended on small filter-paper bridges. Glass wool can also be used to support cultured tissue. Cheng and Voqui[6] have used a fabric support (100% polyester) saturated with a liquid medium for the growth and organogenesis of Douglas fir cotyledon explants. Immersed cultures can be agitated at 1 rpm to 150 rpm to improve aeration. Some cultures, e.g., domestic carrot callus, proliferate faster when only periodically immersed in the medium. This is achieved by the use of a roll-a-drum apparatus which holds test tubes at a 10 to 12° angle as they are rotated.

The quantity of medium used for a particular-sized inoculum of plant tissue may be important. There appears to be a critical cell population of minimum effective density for each tissue.[30] Small quantities of cells in culture often have to be supplied with substances such as amino acids, which are not required by a large mass of cells. This "population effect" may be similar to that reported in pollen germination studies[37,38] in which there appears to be a critical concentration of boron or calcium required. It is possible that cells not only take up nutrients from the culture medium, but also release metabolites into the medium which affect other cells.

h. pH of Nutrient Media

Both the growth-promoting effect and the selectivity of plant tissue culture media are pH dependent. Tissue culture media are usually adjusted to a pH of 5.0 to 6.0 prior to the addition of agar and to autoclaving. In time, the pH of a medium will drift to a neutral pH. Extremes of pH should be avoided as this will block the availability of some nutrients to the tissue. For instance, at both an alkaline pH and at a pH below 3.0, gibberellic acid is activated.[39]

The incorporation of EDTA into media may be important as it maintains the availability of iron and other metal ions as the pH drifts during culture.[30] In sycamore cell suspension cultures, the minimum effective cell density can be reduced by adjusting the pH to 6.4.[30]

B. Optimum Volume of Medium per Culture Vessel

The correct amount of medium for each tissue and vessel type should be tested carefully. Too much medium can adversely affect tissue growth. Conversely, an inadequate quantity of medium can slow growth rates of tissue.

C. Sterilization of Medium

Plant tissue culture media are sterilized by autoclaving or by filter sterilization. The most common technique is to sterilize the media, which has previously been dispensed into culture vessels, at 121°C for 15 min in an autoclave. The use of pressure allows a high temperature to be used without the liquid overboiling. When autoclaved, sucrose in combination with other media constituents can cause a nonenzymatic browning which can be toxic to cultured plant cells.[40]

Media constituents which are heat labile (e.g., gibberellin),[39] can be filter sterilized and added to the remainder of the constituents which have been autoclaved and maintained at 40°C to prevent agar solidification. The heat-sterilized and filter-sterilized components are combined under aseptic conditions in a laminar-flow transfer hood. Filter sterilization can be accomplished by using a membrane filter (millipore), which

is a two-dimensional screen made of homogenous polymers with a uniform pore size. Bacteria and fungi are normally removed with a pore size of 0.45 μm. This technique is particularly useful for the sterilization of heat-labile components.

If desired, all media components can be filter sterilized and the medium dispensed into sterile culture vessels under aseptic conditions. This tends to be time-consuming for large quantities of media, but may be necessary if degradation of media components by heat is suspected.

D. The Culture Environment for Callus

1. Light

Plant-tissue cultures are not photosynthetically efficient and therefore, generally are not autotrophic. Nevertheless, the influence of light on morphogenetic processes within the cultured tissue should not be discounted. When maintaining callus cultures, the tissue should be maintained in darkness to avoid morphogenesis.

2. Temperature

A temperature of 25-27°C is normally employed for in vitro culture. The optimum temperature for the growth of callus cultures may have to be investigated individually for each species.

3. Humidity

The climatic conditions of each area dictate the treatment required by cultures. In tropical, humid regions, dehumidifiers may have to be used in the culture area if high rates of fungal contamination are experienced. When dry atmosphere is encountered, enclosures such as "Kaputs" may have to be used to reduce loss of moisture from both cultures and the medium.

E. Removal or Suppression of Microbial Contaminants from Cultures

Contamination of cultures is a serious problem and can be disastrous when working with valuable material. Attempts to resterilize contaminated tissue by the use of the aforementioned surface sterilants are not usually successful, as the tissue is often damaged. Antibiotics, such as gentamycin, partially suppress bacterial contaminants, but may also reduce growth rates.

IV. REGENERATION OF PLANTS FROM CALLUS CULTURES

Some valuable biochemicals are only synthesized (or synthesized in greater quantity) by differentiated tissue or are only accumulated in specialized organs or tissues. For instance, the total alkaloid content of callus cultures is often low, but is increased with morphogenesis and plant growth.[41] Therefore, it may be necessary to obtain and maintain differentiated and organized tissue for the purpose of extracting drugs. Ideally, one would prefer to obtain chemical diffentiation in suspension cultures, which are easier to grow, without having to resort to morphological specialization. Further, the clonal propagation of a high-yield plant, and its subsequent multiplication by modern nursery techniques, may be at present the most efficient method for the commercial production of a drug. In any event, the principles of regeneration of shoots and plantlets for the two above purposes are similar.

If plant cells are totipotent, that is, capable of regenerating a whole plant from a single cell or small group of cells, then their ability to undergo organogenesis depends on their differentiation to meristematic cells and then their further differentiation to specialized cells.

There are two ways to regenerate plants from callus, through the initiation of shoots