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Low cost options for tissue culture technology in developing countries

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LOW COST OPTIONS FOR TISSUE CULTURE TECHNOLOGY
IN DEVELOPING COUNTRIES

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FOREWORD

Tissue culture technology is used for the production of doubled haploids, cryopreservation, propagating new plant varieties, conserving rare and endangered plants, difficult-to-propagate plants, and to produce secondary metabolites and transgenic plants. The production of high quality planting material of crop plants and fruit trees, propagated from vegetative parts, has created new opportunities in global trading, benefited growers, farmers, and nursery owners, and improved rural employment. However, there are still major opportunities to produce and distribute high quality planting material, e.g. crops like banana, date palm, cassava, pineapple, plantain, potato, sugarcane, sweet potato, yams, ornamentals, fruit and forest trees.

The main advantage of tissue culture technology lies in the production of high quality and uniform planting material that can be multiplied on a year-round basis under disease-free conditions anywhere irrespective of the season and weather. However, the technology is capital, labor and energy intensive. Although, labor is cheap in many developing countries, the resources of trained personnel and equipment are often not readily available. In addition, energy, particularly electricity, and clean water are costly. The energy requirements for tissue culture technology depend on day temperature, day-length and relative humidity, and they have to be controlled during the process of propagation. Individual plant species also differ in their growth requirements. Hence, it is necessary to have low cost options for weaning, hardening of micropropagated plants and finally growing them in the field.

This publication describes options for reducing costs to establish and operate tissue culture facilities and primarily focus on plant micropropagation. It includes papers on the basics of tissue culture technology, low cost options for the design of laboratories, use of culture media and containers, energy and labor saving, integration and adoption of low cost options, and increasing plant survival after propagation, bioreactors, and outreach of material to the growers and farmers in developing countries. Bioreactors in plant propagation can produce millions of plants and may cut down the cost of plant production, which is yet not commonly used in developing countries. However, in the near future it could be well integrated into large scale commercial micropropagation in both developed and developing countries. In all cases, such options must be integrated without reducing the efficiency of plant propagation and compromising the plant quality.

This TECDOC was prepared on the basis of contributions made by the participants in the Technical Meeting on Low Cost Tissue Culture Technology for Developing Countries, Vienna, 26–30 August 2002. This publication should be of great value to plant propagators, managers of tissue culture laboratories, scientists, organizations contemplating the establishment of new laboratories, and ongoing commercial concerns, all of whom may wish to incorporate low cost options into their day-to-day operations. Also, it would greatly serve plant propagation enterprises in developing countries with scarce funds and poor infrastructure for sustainable food production. Many of the options described can also be integrated in tissue culture laboratories that use mutation induction to develop new mutant varieties of both vegetatively and seed propagated plants, and for rapid release of the selected mutants. The IAEA and FAO wish to thank B.S. Ahloowalia, former staff member of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, for compiling and preparing this publication. The IAEA officer responsible for the publication is S. Mohan Jain.

EDITORIAL NOTE

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INTRODUCTION

The large scale commercial propagation of plant material based on plant tissue culture was pioneered in the USA. During the last thirty years, tissue culture-based plant propagation has emerged as one of the leading global agro-technologies. Between 1986 and 1993, the worldwide production of tissue cultured plants increased 50%. In 1993, the production was 663 million plants. By 1997, production had risen to 800 million plants. During 1990–1994, the micropropagation industry declined in Europe, mainly due to production shifting to developing countries, but since then because of the demand for high quality and number, production in European countries has increased. Since 1995, production has increased by 14% in Asian countries, mainly due to the market entry of China, while the increase in South and Central America was from production in Cuba. More recently, some companies from Israel, the USA and UK have shifted their production requirements to Costa Rica and India. Tissue cultured plants have as yet to reach many growers and farmers in the developing countries.

The primary advantage of micropropagation is the rapid production of high quality, disease-free and uniform planting material. The plants can be multiplied under a controlled environment, anywhere, irrespective of the season and weather, on a year-round basis. Production of high quality and healthy planting material of ornamentals, and forest and fruit trees, propagated from vegetative parts, has created new opportunities in global trading for producers, farmers, and nursery owners, and for rural employment.

Micropropagation technology is more expensive than the conventional methods of plant propagation, and requires several types of skills. It is a capital-intensive industry, and in some cases the unit cost per plant becomes unaffordable. The major reasons are cost of production and know-how. During the early years of the technology, there were difficulties in selling tissue culture products because the conventional planting material was much cheaper. Now this problem has been addressed by inventing reliable and cost effective tissue culture methods without compromising on quality. This requires a constant monitoring of the input costs of chemicals, media, energy, labour and capital. In the industrialized countries, labour is the main factor that contributes to the high cost of production of tissue-cultured plants. To reduce such costs, some steps can be partially mechanized, e.g. use of a peristaltic pump for medium dispensing, and of dishwashers for cleaning containers. In the less developed countries of Africa, Asia, and Latin America, where labour is relatively cheaper, consumables such as media, culture containers, and electricity make a comparatively greater contribution to production costs. For example, the cost of medium preparation (chemicals, energy and labour) can account for 30–35% of the micropropagated plant production. However, automated production processes based on pre-sterilized membrane capsules, bioreactors, mechanized explant transfer, and container sealing are not commercially viable propositions in many developing countries. Therefore, low cost alternatives are needed to reduce production cost of tissue-cultured plants.

Many of the low cost technology options described in this publication can be incorporated in various steps of plant micropropagation. The occasional tissue culture-generated variants (somaclones) and rare spontaneous bud mutants as well as those obtained from induced mutations can also be propagated by deployment of low cost techniques described. Micropropagation, in combination with radiation-induced mutations, speeds up the recovery, multiplication and release of improved varieties in vegetatively propagated plants. Hence, low cost technology will be of great value for large scale plant multiplication of mutants of many fruits, shrubs, flowers and forest trees that are conventionally vegetatively propagated.

PLANT TISSUE CULTURE

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Abstract. Plant tissue culture refers to growing and multiplication of cells, tissues and organs of plants on defined solid or liquid media under aseptic and controlled environment. The commercial technology is primarily based on micropropagation, in which rapid proliferation is achieved from tiny stem cuttings, axillary buds, and to a limited extent from somatic embryos, cell clumps in suspension cultures and bioreactors. The cultured cells and tissue can take several pathways. The pathways that lead to the production of true-to-type plants in large numbers are the preferred ones for commercial multiplication. The process of micropropagation is usually divided into several stages i.e., pre-propagation, initiation of explants, subculture of explants for proliferation, shooting and rooting, and hardening. These stages are universally applicable in large-scale multiplication of plants. The delivery of hardened small micropropagated plants to growers and market also requires extra care.

INTRODUCTION

Plant tissue culture refers to growing and multiplication of cells, tissues and organs on defined solid or liquid media under aseptic and controlled environment. Plant tissue culture technology is being widely used for large-scale plant multiplication. The commercial technology is primarily based on micropropagation, in which rapid proliferation is achieved from tiny stem cuttings, axillary buds, and to a limited extent from somatic embryos, cell clumps in suspension cultures and bioreactors.

EXPLANT SOURCE

Plant tissue cultures are initiated from tiny pieces, called explants, taken from any part of a plant. Practically all parts of a plant have been used successfully as a source of explants. In practice, the "explant" is removed surgically, surface sterilized and placed on a nutrient medium to initiate the mother culture, that is multiplied repeatedly by subculture. The following plant parts are extensively used in commercial micropropagation.

*Shoot-tip and meristem-tip culture***:** Shoots develop from a small group of cells known as shoot apical meristem. The apical meristem maintains itself, gives rise to new tissues and organs, and communicates signals to the rest of the plant (Medford, 1992). Shoot-tips and meristem-tips are perhaps the most popular source of explants to initiate tissue cultures. The shoot apex explant measures between 100 to 500µm and includes the apical meristem with 1 to 3 leaf primordia. The apical meristem of a shoot is the portion lying distal to the youngest leaf primordium (Cutter, 1965), and is ca.100µm in diameter and 250µm in length (Quak, 1977) with 800-1200 cells. In practice, shoot-tip explants between 100 to 1000µm are cultured to free plants from viruses. Even explants larger than 1000µm have been frequently

used. The term "meristem-tip culture" has been suggested to distinguish the large explants from those used in conventional propagation (Bhojwani and Razdan, 1983).

Nodal or axillary bud culture: This consists of a piece of stem with axillary bud culture with or without a portion of shoot. When only the axillary bud is taken, it is designated as "axillary bud" culture.

Floral meristem and bud culture: Such explants are not commonly used in commercial propagation, but floral meristems and buds can generate complete plants.

Other sources of explants: In some plants, leaf discs, intercalary meristems from nodes, small pieces of stems, immature zygotic embryos and nucellus have also been used as explants to initiate cultures.

Cell suspension and callus cultures: Plant parts such as leaf discs, intercalary meristems, - stem-pieces, immature embryos, anthers, pollen, microspores and ovules have been cultured to initiate callus. A callus is a mass of unorganized cells, which in many cases, upon transfer to suitable medium, is capable of giving rise to shoot-buds and somatic embryos, which then form complete plants. Such calli on culture in liquid media on shakers are used for initiating cell suspensions. Liquid suspension cultures maintained on mechanical shakers achieve fast and excellent multiplication rates. However, in commercial micropropagation, calli are cultured mostly in bottles and flasks kept on semi-solid or liquid media. To a limited extent, bioreactors (Cf. Chapter 6) have become popular for somatic embryogenic cultures. It is considered that some day robotics could be adapted to bioreactor-based micropropagation.

PATHWAYS OF CULTURED CELLS AND TISSUES

The cultured cells and tissue can take several pathways to produce a complete plant. Among these, the pathways that lead to the production of true-to-type plants in large numbers are the popular and preferred ones for commercial multiplication. The following terms have been used to describe various pathways of cells and tissue in culture (Bhojwani and Razdan, 1983; Pierik, 1989).

Regeneration and organogenesis

In this pathway, groups of cells of the apical meristem in the shoot apex, axillary buds, root tips, and floral buds are stimulated to differentiate and grow into shoots and ultimately into complete plants. In many cases, the axillary buds formed in the culture undergo repetitive proliferation, and produce large number of tiny plants. The plants are then separated from each other and rooted either in the next stages of micropropagation or *in vivo* (in trays, small pots or beds in glasshouse or plastic tunnel under relatively high humidity). The explants cultured on relatively high amounts of auxin (e.g. (2,4-D, 2,4-dichlorophenoxyacetic acid) form an unorganized mass of cells, called callus. The callus can be further sub-cultured and multiplied. The callus shaken in a liquid medium produces cell suspension, which can be sub-cultured and multiplied into more liquid cultures. The cell suspensions form cell clumps, which eventually form calli and give rise to plants through organogenesis or somatic embryogenesis (Cf. Ammirato, 1983). In some cases, explants e.g. leaf-discs and epidermal tissue can also generate plants by direct organogenesis and somatic embryogenesis without intervening callus formation, e.g. in orchardgrass. *Dactylis glomerata* L. (Hanning and Conger, 1986). In organogenesis the cultured plant cells and cell clumps (callus) and mature

differentiated cells (microspores, ovules) and tissues (leaf discs, inter-nodal segments) are induced to differentiate into complete plants to form shoot buds and eventually shoots, and rooted to form complete plants.

Somatic embryogenesis

In this pathway, cells or callus cultures on solid media or in suspension cultures form embryo-like structures called somatic embryos, which on germination produce complete plants. The primary somatic embryos are also capable of producing more embryos through secondary somatic embryogenesis. Although, somatic embryogenesis has been demonstrated in a very large number of plants and trees, the use of somatic embryos in large-scale commercial production has been restricted to only a few plants, such as carrot, date palm, and a few forest trees.

Somatic embryos are produced as adventitious structures directly on explants of zygotic embryos, from callus and suspension cultures. Somatic embryos and synthetic seeds (embryos encapsulated in artificial endosperm) hold potential for large-scale clonal propagation of superior genotypes of heterogeneous plants (Redenbaugh *et al*., 1993; Mamiya and Sakamoto, 2001). They have also been used in commercial plant production and for the multiplication of parental genotypes in large-scale hybrid seed production (Bajaj, 1995; Cyr, 2000). In many species, somatic embryos are morphologically similar to the zygotic embryos, although some biochemical, physiological and anatomical differences have been documented. The synthetic auxin, 2,4-D is commonly used for embryo induction. In many angiosperms, e.g., carrot (Lee *et al*., 2001) and alfalfa (McKersie and Bowley, 1993), subculture of cells from 2,4-D containing medium to auxin-free medium is sufficient to induce somatic embryogenesis. The process can be enhanced with the application of osmotic stress, manipulation of medium nutrients, and reducing humidity. Selection of embryogenic cell lines has also been successfully used. For example, selection for unique morphotypes in grapevine cultures allows production of high quality embryos with predicable frequency (Jayasankar *et al*., 2002).

A major problem in large-scale production of somatic embryos is culture synchronization. This is achieved through selecting cells or pre-embryonic cell clusters of certain size, and manipulation of light and temperature (McKersie and Bowley, 1993), temporary starvation (Lee *et al*., 2001) or by adding cell cycle synchronizing chemicals to the medium (Dobrev *et al*., 2002). Cytokinins seem to play a key role in cell cycle synchronization (Dobrev *et al*., 2002) and embryo induction, proliferation and differentiation (Schuller *et al*., 2000). Abscisic acid is crucial in all the stages of somatic development, maturation and hardening (Schuller *et al*., 2000; Nieves *et al*., 2001).

Synthetic seeds

The concept of production and utilization of synthetic seeds (somatic embryo as substitutes for true seeds) was first suggested by Murashige in 1977 (Cf. Bajaj, 1995; Cyr, 2000). Synthetic seeds can be produced either as coated or non-coated, desiccated somatic embryos or as embryos encapsulated in hydrated gel (usually calcium alginate) (Redenbaugh *et al*., 1993).

Successful utilization of synthetic seeds as propagules of choice requires an efficient and reproducible production system and a high percentage of post-planting conversion into vigorous plants. Artificial coats and gel capsules containing nutrients, pesticides and beneficial organisms have long been thought as substitutes for seed coat and endosperm (Bajaj, 1995). However, this

technology is still in the developmental stage, and currently cannot compete with the other methods of commercial plant propagation (Cyr, 2000).

PROCESS OF MICROPROPAGATION

The process of plant micro-propagation aims to produce clones (true copies of a plant in large numbers). The process is usually divided into the following stages:

Stage 0- pre-propagation step or selection and pre-treatment of suitable plants.
Stage I - initiation of explants - surface sterilization, establishment of mother explants.
Stage II - subculture for multiplication/proliferation of explants.
Stage III – shooting and rooting of the explants.
Stage IV - weaning/hardening.

These stages are universally applicable in large-scale multiplication of plants. The individual plant species, varieties and clones require specific modification of the growth media, weaning and hardening conditions. A rule of the thumb is to propagate plants under conditions as natural or similar to those in which the plants will be ultimately grown *ex-vitro*. For example, if a chrysanthemum variety is to be grown under long day-length for flower production, it is better to multiply the material under long-day length at stages III and IV. There is a wide option to undertake production of plant material up to a limited number of stages. For example, many commercial tissue culture companies undertake production up to Stage III, and leave the remaining stages to others.

Pre-propagation stage

The pre-propagation stage (also called stage 0) requires proper maintenance of the mother plants in the greenhouse under disease- and insect-free conditions with minimal dust. Clean enclosed areas, glasshouses, plastic tunnels, and net-covered tunnels, provide high quality explant source plants with minimal infection. Collection of plant material for clonal propagation should be done after appropriate pretreatment of the mother plants with fungicides and pesticides to minimize contamination in the *in vitro* cultures. This improves growth and multiplication rates of *in vitro* cultures. The control of contamination begins with the pretreatment of the donor plants. They may be prescreened for diseases, isolated and treated to reduce contamination (George, 1993; Holdgate and Zandvoort, 1997). The explants are then brought to the production facility, surface sterilized and introduced into culture. They may at this stage be treated with antibiotics and fungicides (Kritzinger, *et al*., 1997) as well as anti-microbial formulations, such as PPM (Guri and Patel, 1998). The explants are then culture indexed for contamination by standard microbiological techniques, which are occasionally supplemented with tests based on molecular biology or other techniques (George, 1993; Leifert and Woodward, 1998; Leifert and Cassells, 2001).

Stage I

This stage refers to the inoculation of the explants on sterile medium to initiate aseptic culture. Initiation of explants is the very first step in micropropagation. A good clean explant, once established in an aseptic condition, can be multiplied several times; hence, explant initiation in an aseptic condition should be regarded as a critical step in micropropagation. More than often, explants fail to establish and grow, not due to the lack of a suitable medium but because of contamination. The explants are transferred to *in vitro* environment, free from microbial contaminants. The process requires excision of tiny plant pieces and their surface sterilization with chemicals such as sodium hypochlorite, ethyl alcohol and repeated washing

with sterile distilled water before and after treatment with chemicals. After a short period of culture, usually 3 to 5 days, the contaminated explants are discarded. The surviving explants showing growth are maintained and used for further subculture.

In herbaceous plants e.g. potato, chrysanthemum, carnation, streptocarpus, strawberry, and African violet; the explant sources are meristems, apical- and axillary buds, young seedlings, developing young leaves and petioles, and unopened floral buds. The following low cost options can be adapted to initiate explants:

Sterile instrument technique

This method assumes that most of the deep-seated meristems and those covered by leaves or other integuments (e.g. floral bracts) are sterile. In this procedure, the explant is washed with sterile water, rinsed in ethanol, and instruments are sterilized every time they touch the surface of the explant, and the explant is moved to a new location on the dissection stage.

Surface sterilization technique

This is by far the most commonly used method. The explants are washed in sterile water, rinsed in ethanol, and surface sterilization is achieved by using chemicals with chlorine base. Calcium or sodium hypochlorite based solutions, 1-3% (v/v) are usually used for soft herbaceous materials. A cheap and ready-made sterilant is 5-7% solution of 'Domestos'- a toilet disinfectant (Lever Bros. Ltd., UK) which contains 10.5% v/v sodium hypochlorite, 0.3% sodium carbonate, 10.0% sodium chloride and 0.5% (w/v) sodium hydroxide and a patented thickener). The explants are washed in sterile distilled water before and after sterilization. Other surface sterilants used include mercuric chloride (avoid its use as far as possible, since it is highly toxic), hydrogen peroxide, and potassium permanganate. The following protocols are available:

For soft tissues

1. Wash explants from perennial plants for 1-2 hr in tap water. Eliminate this step for material from glasshouse grown plants.
2. Wash in sterile distilled water three to four times for 5 to 10 minutes each.
3. Dip in 95% ethanol for 3 to 5 seconds.
4. Wash once again with sterile distilled water for 5 minutes.
5. Surface-sterilize in 5% 'Domestos' (v/v) for 20-25 minutes.
6. Wash with sterile distilled water three times for 10 minutes each.
7. Drain water droplets by placing on pre-sterilized blotting paper.
8. Transfer explants singly to the medium.

N.B.: Sterilize forceps each time to transfer explants to avoid cross-contamination.

For woody stems (e.g. roses, hardy shrubs, and trees):

1. Collect stems, shoots, buds and store at 5 ^{0}C till needed.
2. Rinse in ethanol for 3 to 5 seconds.
3. Rinse in 1-% sodium hypochlorite (20% bleach) for 10 minutes.
4. Place lower parts of stems in flasks in 2% sucrose and 200 PPM 8-hydroxyquinoline citrate at 23+2 ^{0}C. For items collected in September/October, add 50-PPM GA3. After that l0 PPM GA will help break the dormancy.
5. Re-cut the bottom of stem and replace the solution after 2 days.
6. Excise the softwood from the developed shoots and use material for explants or for rooting.
7. Surface-sterilize as in the above protocol.

Do not forget to sterilize forceps and scalpel every time for the transfer of explants to fresh solutions. Use sterile containers in the protocol of surface sterilization. If explants become brown or pale at the end of the protocol, reduce the strength of 'Domestos' to 2.5%. Alternatively, dip explants in 10% 'Domestos' for 2 minutes and then proceed to surface sterilize with 3-5% 'Domestos' for 20 minutes. If basal contamination is observed after 2-3 days of culture, explants can sometimes be rescued by removing the basal end by making a single cut with a sharp scalpel and re-culturing on fresh medium.

Stage II

Stage II is the propagation phase in which the explants are cultured on the appropriate media for multiplication of shoots. The primary goal is to achieve propagation without losing the genetic stability. Repeated culture of axillary and adventitious shoots, cutting with nodes, somatic embryos and other organs from Stage I leads to multiplication of propagules in large numbers. The propagules produced at this stage can be further used for multiplication by their repeated culture. Sometimes it is necessary to subculture the *in vitro* derived shoots onto different media for elongation.

Stage III

The *in vitro* shoots obtained at Stage II are rooted to produce complete plants. If the proliferated material consists of bud-like structures (e.g. orchids) or clumps of shoots (banana, pineapple), they should be separated after rooting and not before. Many plants (e.g. banana, pineapple, roses, potato, chrysanthemum, strawberry, mint, several grasses and many more) can be rooted on half-strength-MS (Murashige and Skoog, 1962) medium without any growth-regulators (Ahloowalia, *unpublished*). Good sturdy well-rooted plants are essential for high survival during weaning and later transfer to soil. This stage is labour intensive and expensive. The process of *in vitro* rooting has been estimated to account for approximately 35–75% of the total cost of production (Debergh and Maene, 1981). Efforts should be made to combine rooting and acclimatization stages. (Cf. Chapter 9)

Stage IV

At this stage, the *in vitro* micropropagated plants are weaned and hardened. This is the final stage of the tissue culture operation after which the micropropagated plantlets are ready for transfer to the greenhouse. Steps are taken to grow individual plantlets capable of carrying out photosynthesis. The hardening of the tissue-cultured plantlets is done gradually from high to low humidity and from low light intensity to high intensity conditions. If grown on solid medium, most of the agar can be removed gently by rinsing with water. . Plants can be left in shade for 3 to 6 days where diffused natural light conditions them to the new environment. The plants are then transferred to an appropriate substrate (sand, peat, compost, etc.), and gradually hardened. Low-cost options include the use of plastic domes or tunnels, which reduces the natural light intensity and maintains high relative humidity during the hardening process. If the plants are still joined together after rooting, these should be planted as bunches in the soil and separated after 6 to 8 weeks of growth.

DELIVERY TO THE GROWERS

The delivery of the rooted and hardened small micropropagated plants to growers and market requires extra care. In some cases, plant losses can occur during shipment and handling by growers. This is particularly true when the plants are not fully hardened and

rooted or not grown for sufficient duration after transfer to soil. Growers should be given clear instructions how to handle the material provided. Apart from the economic loss, poor survival of planted material erodes the confidence of growers in the technology. The transfer of individual plants to soil in black plastic or polythene bags is widely used as a low-cost option to provide fully-grown banana plants directly to farmers in many developing countries.

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ROLE OF LOW COST OPTIONS IN TISSUE CULTURE

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Abstract. The primary application of micropropagation has been to produce high quality planting material Low-cost tissue culture technology is the adoption of practices and use of equipment to reduce the unit cost of micropropagule and plant production. Low cost options should lower the cost of production without compromising the quality of the micropropagules and plants. In low cost technology cost reduction is achieved by improving process efficiency and better utilization of resources. Low-cost tissue-culture technology will stay a high priority in agriculture, horticulture, forestry, and floriculture of many developing countries for the production of suitably priced high quality planting material.

INTRODUCTION

Hundreds of commercial micropropagation laboratories worldwide are currently multiplying large number of clones of desired varieties and local flora. Apart from the rapid propagation advantage, this technology is being used to generate disease-free planting material, and has been developed and applied to a wide range of crops, and forest and fruit trees. However, in many cases, the cost of micropropagule production precludes the adoption of the technology for large-scale commercial propagation.

THE NEED FOR LOW COST TECHNOLOGY

Low-cost tissue culture technology is the adoption of practices and use of equipment to reduce the unit cost of micropropagule and plant production. In many developed countries, conventional tissue culture-based plant propagation is carried out in highly sophisticated facilities that may incorporate stainless steel surfaces, sterile airflow rooms, expensive autoclaves for sterilization of media and instruments, and equally expensive glasshouses with automated control of humidity, temperature and day-length to harden and grow plants. Many such facilities established at a high cost are high-energy users, and are run like a super-clean hospital. The requirements to establish and operate such tissue culture facilities are expensive, and often are not available in the developing countries. For example, the cost of electricity in the developed countries is much lower, and its supply far better assured than in the developing countries. The same can be said of the supply of culture containers, media, chemicals, equipment and instruments used in micropropagation. Hence, alternatives to expensive inputs and infrastructure have been sought and developed to reduce the costs of plant micropropagation.

ADOPTION OF LOW-COST OPTIONS

Low cost options should lower the cost of production without compromising the quality of the micropropagules and plants. The primary application of micropropagation has been to produce high quality planting material, which in turn leads to increased productivity in agriculture. The generated plants must be vigorous and capable of being successfully transplanted in the field, and must have high field survival. In addition, they should be genetically uniform, free from diseases and viruses, and price competitive to the plants produced through conventional methods. Reducing the cost should not result in high contamination of cultures or give plants with poor field performance.

The foremost requirement of micropropagation is the aseptic culture and multiplication of plant material. Microbe-free conditions need to be maintained in culture containers, and during successive subcultures. In many cases, mistakes in concept or practice can introduce microbes in the culture containers from an external source or the plant material itself (endophytic contamination). As a result, the microbes overgrow the cultures, and wipe them out. Microbes may grow slowly under controlled low temperature, but they proliferate very fast under uncontrolled and high temperature. Thus the adoption of wrong low-cost options may make the production process prone to disasters. Low cost techniques will succeed only if the basic conditions for tissue culture are scrupulously adhered to maintain propagule quality.

Microbial contamination of cultures is known to wipe out work of months, and can turn into a nightmare. The best low-cost option is to discard and dispose of contaminated cultures outright. Avoiding contamination in small R&D laboratories is not a difficult task where only a low number of cultures are handled. However, commercial production involves handling of thousands of cultures each day. It is also essential to maintain such cultures in large numbers under contamination-free conditions, until they are used for either further subculture or hardening and growing-on. Plants do not have an immune system and there is a limitation on the use of antibiotics to circumvent the problem. Moreover, many of the antibiotics, which are effective against bacteria, fungi, and phytoplasmas, are toxic to plants as well. Obviously, the use of antibiotics is not foolproof or the desired method to rid microbial contamination (Pierik, 1989). Sophisticated state-of-the-art facilities are not a guarantee for prevention of contamination. The laboratories that succeed in an immaculate control of contamination do so by adherence to scrupulous techniques of basic tissue culture. Thus, it is not the sophistication but the procedures that ensure the quality of tissue cultured plants.

QUALITY OF MICROPROPAGULES

Low cost technology means an advanced generation technology, in which cost reduction is achieved by improving process efficiency, and better utilization of resources. Presently, both the developing and the developed countries require low cost technology to progressively reduce the cost of propagule production. In many developing countries, the potential end-users of plants derived from tissue culture have been the resource-rich farmers. They know the benefits and potential of healthy planting material. Such growers are prepared to risk investment in the high productivity potential of the planting material. For example, hybrid seeds of many vegetables, papaya, rice, and cotton cost 15-20 times more than the price of ordinary varieties. Yet there is a wide market for them. Hence, the production of low quality plants, just because they are less costly, is not going to be a sustainable approach for the application of micropropagation in agriculture. Lowering of cost of production is possible only if the methods do not compromise the basic imperatives of tissue culture and quality of plants.

IMPORTANCE OF LOW COST TECHNOLOGY

The potential of plant tissue culture in increasing agricultural production and generating rural employment is well recognized by both investors and policy makers in developing countries. However, in many developing countries, the establishment cost of facilities and unit production cost of micropropagated plants is high, and often the return on investment is not in proportion to the potential economic advantages of the technology. These problems can be addressed by standardizing agronomic practices more precisely (precision agriculture) and by achieving maximum net profits from the crops or by decreasing the unit cost of production or both. The technology is particularly relevant to the propagation of

ornamental plants. Despite high costs of production, trading of ornamental plants has thrived because they command high unit value. However, the market is limited. Over a period of time, many new tissue-culture companies in several developing countries have entered to compete in the limited market. The inevitable result has been the reduction of net margins below viable limits.

Many international organizations also agree that tissue culture technology is very relevant to agriculture, provided the problem of high cost of production is satisfactorily solved. The role of tissue culture was clearly recognized by the FAO (1993) in the paper on 'Biotechnology in agriculture, forestry and fisheries - FAO's Policy and Strategy'. The report pointed to the wide use of tissue culture techniques for multiplication of elite clones and elimination of pathogens in planting material. It also pointed to the successful tree regeneration in about 100 forest species and its value for breeding, clonal testing and rapid deployment of superior genotypes.

Several R&D projects have been undertaken to improve the productivity of agricultural, horticultural and forest trees by the European Union under Co-operation in the Field of Scientific and Technical Research (COST). Under this program, coordinated and funded by the European Union, one of the primary aims has been to reduce micropropagation cost. For example, the objective of 'COST 843' action has been the innovation of low-cost plant propagation methods that enhance sustainable and competitive agriculture and forestry in Europe (COST Action, 2001). The high costs of labour of micropropagation are a major bottleneck in the EU to fully exploit *in vitro* culture technology. In the EU, labour currently accounts for 60-70% of the costs of the *in vitro* produced plants. In another program, the large-scale production and introduction of bamboo in the EU using tissue culture technology has been undertaken with the main objective of reducing the costs of micropropagation.

FUTURE ROLE

It has been stressed time and again that in the long-term agriculture and forestry need to be sustainable, use little or no crop-protection chemicals, have low energy inputs and yet maintain high yields, while producing high quality material. Biotechnology-assisted plant breeding is an essential step to achieve these goals.

Plant tissue culture techniques have a vast potential to produce plants of superior quality, but this potential has been not been fully exploited in the developing countries. During *in vitro* growth, plants can also be primed for optimal performance after transfer to soil (Cf. Chapter 9). In most cases, tissue-cultured plants out-perform those propagated conventionally. Thus *in vitro* culture has a unique role in sustainable and competitive agriculture and forestry, and has been successfully applied in plant breeding, and for the rapid introduction of improved plants. Bringing new improved varieties to market can take several years if the multiplication rate is slow. For example, it may take a lily breeder 15-20 years to produce sufficient numbers of bulbs of a newly bred cultivar before it can be marketed. *In vitro* propagation can considerably speed up this process. Plant tissue culture has also become an integral part of plant breeding. For example, the development of pest- and disease-resistant plants through biotechnology depends on a tissue culture based genetic transformation. The improved resistance to diseases and pests enables growers to reduce or eliminate the application of chemicals.

The FAO Committee on Agriculture has perceived plant tissue culture as a main technology for the developing countries for the production of disease-free, high-quality planting material, and its commercial applications in floriculture and forestry (FAO, 1999). It further points out that tissue culture techniques are being used particularly for large-scale plant multiplication. Micropropagation has proved especially useful in producing high quality, disease-free planting material for a wide range of crops. Tissue cultured based industry also generates much-needed rural employment, particularly for women.

In forestry, the availability of tissue culture linked production systems may effectively provide sustainable alternatives to the need for harvesting wood from native forests and natural habitats. Successful protocols now exist for the micropropagation of a large number of forest tree species, and the number of species for which successful use of somatic embryogenesis is increasing. Thus in the future, it is likely that micropropagation in the forestry sector will become commercially important. Compared to vegetative propagation through cuttings, the high multiplication rates available through micropropagation offer a much quicker capture of genetic gains obtained in forest tree breeding programs. However, the current high costs will also be one of the major impediments to the direct use of micropropagation in many programs.

The broad application of existing technologies to plantation species is important for tree improvement in the tropics (Haines and Martin, 1997). In a small number of plantation programs, micropropagation is being used as an early rapid multiplication step. However, it has been pointed out that the current high costs will be an impediment to the direct use of micropropagules as planting stock. Micropropagation clearly has a role, in the rapid multiplication of the selected clones for conventional production of cuttings. The direct use of micropropagules as planting stock in industrial plantation can dramatically broaden forestry tree farming if propagation costs are reduced.

The availability of micropropagation technologies will also be useful in genetic engineering applications, e.g., the production of plants as a source of “edible” vaccines. There are many other useful plant-derived substances which can be produced in tissue cultures, sometimes more cheaply and reliably than from natural forests and plantations. These include medicinal compounds and drugs now being sought in major prospecting operations in the tropical forests.

Micropropagation has been identified as a suitable technology in the development projects of UNESCO in Africa and the Caribbean; however, the cost of production must be reduced (Brink *et al*., 1998). Practically in all developing countries, the private industry is the most important group that requires cost-effective technology. For example, in India of the 90 commercial micropropagation units established initially, 32 were closed down. Of those engaged in commercial production, many are uneconomic mainly due to the high cost of production (Anonymous, 2002) and the absence of quality tests. Hence, low-cost tissue-culture technology will stay a high priority in agriculture, horticulture, forestry, and floriculture of many developing countries.

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PHYSICAL COMPONENTS OF TISSUE CULTURE TECHNOLOGY

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Abstract. The physical components of a typical plant tissue culture facility include equipment and buildings with preparation room, transfer room, culture or growth room, hardening and weaning area, soil-growing area (greenhouses, plastic tunnels), packaging and shipping area, and related facilities – office and store for chemicals, containers and supplies. Careful planning is the first important step when considering the size and location of a facility. The size of the physical components of a tissue culture facility will vary according to its functional needs – the volume of production. It is recommended that an existing facility should be visited to view the layout and operational needs before starting a new facility. A number of low-cost alternatives can be used to simplify various operations and reduce the costs in a tissue culture facility.

INTRODUCTION

In designing any laboratory, big or small, certain elements in its design and layout are absolutely essential for its successful operation. Correct design of a laboratory will not only reduce contamination, but also achieve a high efficiency in work performance (Bridgen and Bertok, 1997). Properly planned and designed laboratories can reduce both the operational and energy costs. A tissue culture laboratory must be designed to accommodate the equipment and its use in the various stages of micropropagation in the most efficient manner. This chapter describes planning the functional needs of an average-sized micropropagation facility. The information given below would allow better planning and stream-lined functioning of the facility, and thereby lead to cost reduction.

General consideration for location

A convenient location for a small laboratory can be a room or part of the basement of a house, a garage, a remodeled office or a room in the header house. The minimum area required for media preparation, transfer and primary growth shelves is about 14 m^2. Walls should be installed to partition different areas. Before setting up a commercial micropropagation unit, it is essential to check out the area keeping in mind the climate, and access to water, electricity, transportation, and infrastructure for supplies. A temperate climate is usually better suited to tissue culture ventures. This greatly reduces the cost of cooling required to maintain the temperature for optimum growth of the cultures.

The availability of electricity and water is of utmost importance, and should be taken into consideration while choosing the location of the facility. For example in India, of the 76 commercial tissue culture units, nearly 52 are located in and around the cities of Bangalore and Pune, where the climate is moderate. Hence, cooling is required only during certain periods of the year. However, in cities like Delhi, which have extremes of climate, tissue culture facilities require both heating and cooling. In a facility, which produces five million plants, the electricity cost per thousand plants is around US $0.30 in Bangalore and Pune; the same is about US $0.80 in Delhi. Disruption of power and water supply causes major

breakdown in the smooth running of tissue culture units. Poor quality water adds to the cost of media.

Location consideration should also include a check with local authorities about zoning and building permits before construction begins. The buildings should be located away from sources of contamination such as a gravel driveway, parking lot, soil mixing area, shipping dock, pesticide storage, and dust and chemicals from fields. Sanitation of the area is important in selecting location.

The units should be located in areas where insect populations are minimal and air has low dust and pollen counts. In the case of export-oriented units, these should be near an international airport, which will reduce the time lag between packaging and shipment. This is critical to assure timely delivery of quality tissue culture products. For new ventures, the size of the facility should be kept small until the market acceptance is ensured.

Design and lay out of the laboratories

Plant tissue culture laboratories have specific design requirements. Careful initial planning is, therefore, a prerequisite for successful running of a facility. The location and design of the laboratories should take into account isolation from foot traffic, control of contamination from adjacent rooms, thermostatically controlled heating and cooling, water supply and drains for a sink, adequate electrical service, provisions for a fan and intake blower for ventilation, and good lighting. Large sized facilities are frequently built free standing. Although more expensive to build, the added isolation from adjacent activities keeps the laboratory clean. Prefabricated buildings make convenient low-cost laboratories. They are readily available in various sizes in many countries. Prefabricated buildings assembled on site can also be used. A single span building allows for a flexible arrangement of walls for dividing into convenient sized rooms.

The floor should be of concrete or capable of carrying 170.5 kg/m^2 (50 pounds per sq. foot). Walls and ceiling should be insulated to at least R-15, and covered inside with water-resistant material. Windows, if desired, may be placed wherever convenient in the media preparation and glassware washing rooms. The heating system should be capable of maintaining room temperature at 20^0C during the coldest part of winter. A minimum of 2cm pipes should be used for water supply. Connection to a septic system or sanitary sewer should be provided. Air conditioning requirements should carefully estimated. Electrical service capacity for equipment, lights and future expansion should be calculated. For safety reasons, the electrical installation should be carried out professionally. Most electrical wiring will require 220 Volts, and autoclaves 230/250 Volts.

The areas such as the media preparation room, inoculation room and growth chambers should be isolated as 'clean zones' (Broome, 1986). The office, storage area, staff centre and packaging rooms can be maintained under ordinary conditions. The working areas must be demarcated according to the activities involved in the facility. Cleanliness is the major consideration when designing a plant tissue culture laboratory to minimise contamination. A positive pressure module should be installed to circumvent air intake from outside. Routine cleaning and aseptic procedures can decrease contamination losses to less than 1%. An enclosed entrance should precede the laboratories, and sticky mats should be placed to collect dirt from shoes.

The traffic pattern and workflow in the laboratory must be considered to maximise cleanliness. The cleanest rooms or areas are the culture room (aseptic transfer area) and the growth room. There should be no direct access to these rooms from outside. The media preparation area, glassware washing and storage areas should be located away from these rooms. The growth room and aseptic transfer rooms should be fitted with see-through doors and should be adjacent to each other. Traffic through these areas should be minimal and restricted to the personnel working under laminar flow cabinets. Ideally, the media preparation area leads into the sterilisation area, which leads into the aseptic transfer room, and eventually to the growth room. Temperature and fire alarms must be connected directly to telephone lines to give fast warnings. An emergency generator should be available to operate essential equipment during power breakdown.

ESSENTIAL EQUIPMENT

The basic equipment in most tissue culture facilities includes the following:

Autoclave

An autoclave is basically a large-sized but sophisticated pressure cooker, and is used for the sterilisation of the medium, glassware and instruments. Autoclaves of different sizes are available commercially. High-pressure heat is needed to sterilise media, water, and glassware. Certain spores from fungi and bacteria are killed only at 121^0C and 1.05kg/sq.cm (15 pounds per sq. inch) pressure. Self-generating steam autoclaves are more dependable and faster to operate.

Laminar airflow chamber

The laminar flow chambers provide clean filtered air that allows cultures to be handled under contamination-free environment. Several types of laminar flow chambers are sold on the market and are available in different sizes. The laminar-flow cabinets are located in the culture transfer area. Some large-sized laboratories have sterile rooms in addition to laminar flow cabinets.

Other equipment

The sterilisation of instruments, such as the forceps, scalpel holders and blades is achieved with either gas flamed burners or with glass-bead sterilizers. The medium preparation room usually has the following equipment. A refrigerator-freezer to store chemicals and stock solutions, weighing scales for large amounts of over 10 g, and an analytical balance with1 mg accuracy, a magnetic stirrer for the agitation, and a pH meter. Small laboratories may locate the refrigerator under the workbench to save space. High quality balances are essential in a tissue culture laboratory. Most laboratories have top loading balances, which allow quick and efficient weighing. A hot plate with an automatic stirrer is needed to for preparing the media before autoclaving. The pH meter is needed to determine pH of the media. Some laboratories use pH indicator paper, however this method is considerably less accurate, and can severely affect the results. An aspirator can be attached to a water tap for filter sterilisation of chemicals and for surface sterilisation of the plant material. However, vacuum pumps are faster and more efficient, but also more expensive.

A drying oven is required to keep glassware such as beakers, flasks and cylinders, and is also useful for dry sterilisation of scalpels and glassware, such as Petri dishes, pipettes and

others. The media containing carbon sources (e.g. sugars) and growth regulators are sterilised in the autoclave, but sometimes, aseptic filtration is better to avoid breakdown of heat-labile chemicals. The water still is also located in the medium preparation area. To prepare media, distilled or de-ionised water is generally used, although tap water can be used in some cases (Cf. Chapter 4).

OPTIONAL EQUIPMENT

A variety of non-essential equipment is used in tissue culture laboratories. The specific requirements determine what need to be purchased. Microwave ovens are convenient for defrosting stock solutions and pre-heating agar media. Most laboratories have a dissecting microscope to excise small explants. Laboratory glassware washers or regular dishwashers can be used for replacing manual labour. Automatic media dispensers are helpful to pipette pre-set volume of media. A gyratory shaker or a reciprocal shaker is necessary if micropropagation is based on liquid media or suspension cultures. Computers, photocopiers and fax machines are helpful for easy data management and maintenance of records. Some of the equipment may be costly, but goes a long way in saving time and labour and is essential for rapid communication in the competitive world.

ACTIVITY SPECIFIC REQUIREMENTS

Based on the different activities of a tissue culture, a facility can be divided into semi-clean, clean and ultra-clean areas. The semi-clean areas comprise of the washing room, office and staff restrooms, where there is no need for maintaining sterile conditions. The clean areas encompass the media preparation and sterilisation rooms, which have to be sufficiently clean. High sterility has to be maintained in the culture transfer rooms and the growth rooms, which constitute the ultra-clean areas.

Glassware washing and storage area

The glassware washing area should be located near the sterilisation and medium preparation rooms. This area should have at least one large sink but two sinks are preferable. Adequate workspace is required on each sides of the sink; this space is used for glassware soaking and drainage. Plastic netting can be placed on surfaces near the sink to reduce glassware breakage and enhance water drainage. The outlet pipe from the sink should be of PVC to resist damage from acids and alkalis. Both hot and cold water should be available and the water still and de-ionisation unit should be located nearby. The choice of electrical washers should be based on the projected use, durability, reliability and cost, and service availability. In India and some other developing countries, where labour is relatively cheap, washing is done manually. The washing room should be swapped periodically. Mobile drying racks can be used and lined with cheesecloth to prevent water dripping and loss of small objects. Ovens or hot air-cabinets should be located close to the glassware washing and storage area. Dust-proof cabinets and storage containers should be installed to allow for easy access to glassware. When culture vessels are removed from the growth area, they are often autoclaved to kill contaminants and to soften semi-solid media. It should be possible to move the vessels easily to the washing area. The glassware storage area should be close to the wash area to expedite storage and access for media preparation.

The store

It is advisable to have a separate area for storage of chemicals, apparatus and equipment. It would not only facilitate constant availability but also save cost from bulk purchase. Chemicals required in small amounts should not be purchased in large quantities as they may lose their activity, pick up moisture or get contaminated (George, 1993). Such problems can be overcome by purchasing small lots on a regular basis.

Media preparation and sterilisation area

The media preparation room should have smooth walls and floors, which enable easy cleaning to maintain a high degree of cleanliness. Minimum number of doors and windows should be provided in this room but within the local fire safety regulations. This reduces cost and contamination. The media preparation and sterilisation can be carried out in the same area but preferably in different rooms, which need not be separated with doors. Media preparation area should be equipped with both tap and purified water. An appropriate system for water purification must be selected and fitted after careful consideration of the cost and quality. A number of electrical appliances are required for media preparation; hence, it is essential to have safety devices like fire extinguisher, fire blanket and a first aid kit in the media preparation room. A variety of glassware, plastic ware and stainless steel apparatus is required for measuring, mixing, and media storage. These should be stored in the cabinets built under the worktables and taken out for use as and when required. This would save the cost and space for building storage shelves. The use of glassware should be kept at a minimum, as it will help in reducing losses due to breakage. As far as possible, plastic ware and stainless steel vessels should be used, as they are much cheaper and more durable than glassware. The water source and glassware storage area should be in or near the medium preparation area. Work bench tops, suitable for comfortable working while standing should be 85 to 90 cm high and 60 cm deep. The workbench tops should be made with plastic laminate surfaces that can tolerate frequent cleaning.

Sterilisation room

The sterilising room should be in continuation with the media preparation room. The layout must be planned in such a way that it ensures the smooth movement of the containers from the washing to the media preparation and sterilisation room. The sterilisation room must have walls and floors that can withstand moisture, heat and steam. An exhaust should be fitted to remove the warm and moist air. The exhaust fan should have an outer cover to prevent entry of outside air. The fan cover should open only when the fan is in operation. In small tissue culture facilities, costly autoclaves can be replaced by simple pressure cookers. However, for large volume media making, horizontal or vertical autoclaves should be installed. Double door autoclaves, which open directly into the media storage room, may be costly but reduce contamination. A cheaper alternative is to transfer the sterilised media to the adjoining room through a hatch window.

Transfer room

The most important work area is the culture transfer room where the core activity takes place. The transfer area needs to be as clean as possible and be a separate room with minimal air disturbance. Walls and floors of the transfer room must be smooth to ensure frequent cleaning. The doors and windows should be minimal to prevent contamination, but within local safety code. There is no special lighting requirement in the transfer room. The

illumination of the laminar airflow chamber is sufficient for work. Sterilisation of the instruments can be done with glass-bead sterilizers or flaming after dipping in alcohol, usually ethanol. The culture containers should be stacked on mobile carts (trolleys) to facilitate easy movement from the medium storage room to the transfer room, and finally to the growth room. The chair seats of the transfer operators should be comfortable, as they have to work for long periods in the same place. Fire extinguishers and first aid kits should be provided in the transfer room as a safety measure. The personnel should leave shoes outside the room. Special laboratory shoes and coats should be worn in this area. Ultraviolet (UV) lights are sometimes installed in transfer areas to disinfect the room; these lights should be used only when people and plant material are not in the room. Safety switches can be installed to turn off the UV lights when regular room lights are turned on.

Growth room

Growth room is an equally important area where plant cultures are maintained under controlled environmental conditions to achieve optimal growth. It is advisable to have more than one growth room to provide varied culture conditions since different plant species may have different requirements of light and temperature during *in vitro* culture. Also, in the event of the failure of cooling or lighting in one room, the plant cultures can be moved to another room to prevent loss of cultures. In the growth room, the number of doors should be minimal to prevent contamination. There is no need for windows in the growth room, except when natural light is used. When artificial lighting is used, the external light can interfere with the photoperiod and temperature of the growth room. Depending on the amount of available space and cost, the culture containers can be placed on either fixed or mobile shelves. Mobile shelves have the advantage of providing access to cultures from both sides of the shelves. The height of the shelves should not exceed 2m. High shelving requires step-up stools to place and remove cultures, being dangerous and time consuming. The primary source of illumination in the growth room is normally from the lights mounted on the shelves. Overhead light sources can be minimised, as they would be in use only while working during the dark cycle. Plant cultures may not receive uniform light from the conventional downward illumination. Lights directly fitted to the racks create uneven heat distribution. This leads to high humidity within the culture containers, which in turn can cause hyperhydricity. Sideways illumination is an alternative, which requires less number of lights, and provides more uniform lighting. But care has to be taken not to break the lights while moving the cultures across the shelves.

CONTROL OF GROWING CONDITIONS

Controlled temperature, lighting and relative humidity, and shelving need to be considered in planning the growth room. These vary depending on the size of the growth room, its location, and the type of plants cultured. For example, a small growth room located in the cool North American climate can be placed in an unheated or minimally heated basement. The chokes (ballasts) of the fluorescent lights need not to be separated; rather they can serve as a source of heat. Excess heat can be dissipated from the growth room, and used for heating other areas in the basement. In such a situation, solid wooden shelves with space between shelves can be used and prevent culture vessels on shelf above the lights from becoming over-heated. However, a large growth room located above ground needs to have the light chokes installed outside the room. Shelves in large growth room can be of glass or metal wire mesh.

Temperature control

Temperature is a primary concern in growth rooms; it affects decisions on installation of lights, control of relative humidity, and type of shelving. Temperature in the growth room is usually controlled with air conditioners. Generally, temperatures are kept around 22^0C. Heating is provided from conventional heating systems and can be supplemented with heat from light chokes. In most developing countries, cooling the growth room is usually a bigger problem than heating. Cooling can be provided with heat pumps, air conditioners and exhaust fans. Using open windows to cool culture rooms leads to contamination during summer and humidity problems in winter.

Lighting control

Some plant cultures can be kept in complete darkness; however, most culture rooms need to be illuminated at 1 Klux [134.5 µmole/m^2/s (microeinsteins per second per sq. centimetre or approximately 1076 foot candle) with some up to 5 to 10 Klux (672-1345 µmole/m^2/s). The plant species and/or propagation scheduling determines the light intensity. The developmental stage of the plants also determines if wide spectrum or cool white-fluorescent lights are to be used. Rooting is strongly influenced positively with far-red light; therefore, wide spectrum lights should be used during Stage III and cool-white lights during Stage I and II. Automatic timers are needed to maintain the desired photoperiod. Reflectors can be placed over bulbs to direct the light downwards and evenly. Heat generated by lights may cause condensation and temperature problems. Small fans placed at the end of the shelves increase airflow and decrease heat build-up. Reflective glossy paint on the walls provides an even light distribution as well as reduces the number of lights required. Relative humidity (RH) is difficult to control inside the culture vessels, but wide fluctuations in the growth room have a deleterious effect. Cultures can dry out if the room's RH is less than 50%. Humidifiers can be used to correct this problem. If the RH becomes too high, a dehumidifier is recommended.

Shelving

Shelves within the growth rooms vary depending upon the situation and the plants grown. Frames for the shelves can be made from 1.25cm (half-inch) thick angle iron. Shelves built from rigid wire mesh to allow maximum air movement and minimise shading should be used. Wood is inexpensive to build shelves. The wood for shelving should have smooth exterior, and should be painted white to reflect light. Expanded metal is more expensive than wood, but provides better air circulation. Tempered glass is sometimes used for shelves to increase light penetration, but it is more prone to breaking. Air spaces of 5 to 10cm between the lights and shelves decrease heat on upper shelves and reduce condensation in culture vessels. A room that is 2.4m high will accommodate 5 shelves, each 45cm. apart, when the bottom shelf is 10 cm above the floor.

GREENHOUSE FACILITY

A critical stage in plant tissue culture is the interim phase between the laboratory and field conditions. *In vitro* derived plants need to be gradually hardened to field conditions (Cf. Chapter 9). Plant hardening is usually carried out under greenhouse that ensures high survival of the tissue-cultured plants in the field. There are three types of greenhouses: Ground to ground, Gable, and Quonset type. The most commonly used greenhouse is the Quonset type. It contains movable or fixed benches with hardening tunnels on them. The size of the greenhouse must be based on the scale of production. Greenhouse glazing can be of glass or

fibreglass. Polyethylene films or sheets of polycarbonate or acrylic can also be used. Air inflated double polyethylene covering is the most economic (Table 1). Appropriate light, shading and blackout systems can be achieved with supplementary lighting. Drip irrigation systems, misting and fogging can be installed as needed. Greenhouses erected in warm climates should have fan-assisted drip pad cooling especially during summer (Broome, 1986). Greenhouses in colder climates need to be heated. Floor and bench systems can be used for heating and cooling the air. Low cost plastic pipes can be used to circulate warm air, which are adequate and cost effective.

PACKAGING AND SHIPPING

A separate area should be designated for packaging in a commercial tissue culture unit. Packaging materials such as cardboard cartons and labels should be stored in this area. The type of packaging of a particular plant depends greatly on the temperature zones through which the consignment has to pass from the point of shipment to its destination. For example, if the consignment of tissue-cultured plants is passing anywhere within temperate zones, it is only necessary to take care of the frost conditions and pack accordingly. But if the consignment is passing from a tropical country to a temperate country, it becomes necessary to take care of the temperature zones in different places through which the consignment passes. Thus, the type of packaging of tissue-cultured products varies with plant and destination. For example, in a package of 22 kg, about 40,000 lily plantlets can be packed. However, in the same parcel, only 20,000 *Spathiphyllum* plantlets or 10,000 *Syngonium* plantlets can be packed because of the higher respiration rates in these species. The heat build-up is much more in *Spathiphyllum* and *Syngonium* plants than the dormant lilies. Similarly, only 8000 *Gerbera* or 7000 *Cordyline* plants can be packed in the same package. Cooling material such as ice, dry ice and other material take up much of the packaging space.

Before loading and shipping, the packed items should be properly counted and rechecked. The cartons containing the cultures for shipping to the customer should be properly labelled with the names and addresses of the consignor and the consignee, and the details of the commodity (storage temperature, handling, etc.). Adequate care must be taken while packaging large consignments, so that there is no disturbance or damage during transit. To prevent any sort of delay, ensure that the consignment is accompanied by documents such as invoice, packing list, import permit, phytosanitary certificate and Generalized System of Preferences (GSP).

OFFICE AND STAFF AREA

In any commercial tissue culture unit, an office is a prerequisite to manage the facility. The office should be large enough to accommodate a filing cabinet, computer, a photocopier and a fax machine for keeping records of cultures, shipments and to handle correspondence. Other activities like maintaining inventory, ordering fresh supplies and accounting can also be done in the office area. In a moderately sized production unit, office area can also double as reception area.

The employee must be provided with changing room and rest room (washroom). The latter are absolutely essential in the event of spills. Tables and chairs must be provided for tea and lunch breaks outside the laboratory area. The staff area must also have lockers, fire extinguishers and first-aid supplies. Food and drink vending machines can also be provided.

Table 1. Cost comparison of a hardening glasshouse and low-cost alternative*

Hardening glasshouse	Cost US$	Low cost alternative	Cost US$
8.8 x 29.2 m fibreglass greenhouse with fan and pad cooling system	30000	8.8 x 29.2 m double polyhouse with air inflation and cooling facility	15000
Misting	3000	Misting	not required
Fogging	5000	Fogging	not required
Benches	6000	Shading	not required
Shading	5000	Tunnels	not required
Tunnels	5000	Benches	6000
		Grow lights	3000
		Clean air filter	1500
Total	54000	Total	24000

*Adapted from Prakash, 1996.

COST OF FACILITY

The building of a tissue culture facility includes the cost of land, construction, electrical installation and plumbing. If the available funds are limited, well-designed laboratories can be established by modifying existing structures. For example, a two or three room house or a trailer with appropriate modifications can be converted into a medium sized micropropagation facility (Fig.1). Depending on the production and storage capacity, a facility can be small- less 100,000 plants, medium- 100,000 to 500,000 plants, or large-scale- 500,000 to 2,000,000 plants per annum.

The initial investment required for setting up a low cost, medium scale tissue culture laboratory (ca. 195 m^2) in India is given in Table 2. Land is sold at a premium, especially near large cities. In India, the land and building costs work out to about US $62500. A house, ca. 195 m^2, can be converted into a tissue culture facility with a capacity of 200,000 plants. The rental charges for such a structure would be around US $5000 per year. The initial investment would thus include only plumbing and electrical work, reducing the capital cost to US $2750. Thus, in large cities, it would be more economic to rent than build the premises (Table 2). If the hardened tissue-cultured plants were the end products, an additional investment of US $20,000 would be required to erect a greenhouse. If the production is limited to-in-agar and ex-agar products (rooted or non-rooted micro-cuttings), greenhouse would not be required.

Table 2. Cost of setting up a tissue culture facility in India

Requirement	Cost (US $)	
	Rented building	Purchased building
Infrastructure	2750	62,437
Equipment	13885	13885
Accessories	4193	4193
Furniture	7333	7333
Miscellaneous	521	521
Total	28,682	88,369

The basic equipment will cost US $14000. The consumables, such as glassware, chemicals and disposable items would require another US $4200. Besides the fixed capital, a commercial tissue culture unit has the recurring expenses of rent, building maintenance, electricity and overheads. The estimated cost of furniture that includes racks with illumination source for growth room, cupboards, benching and chairs would be US $7400. Other miscellaneous items such as labelling and sealing machines would cost US $50.

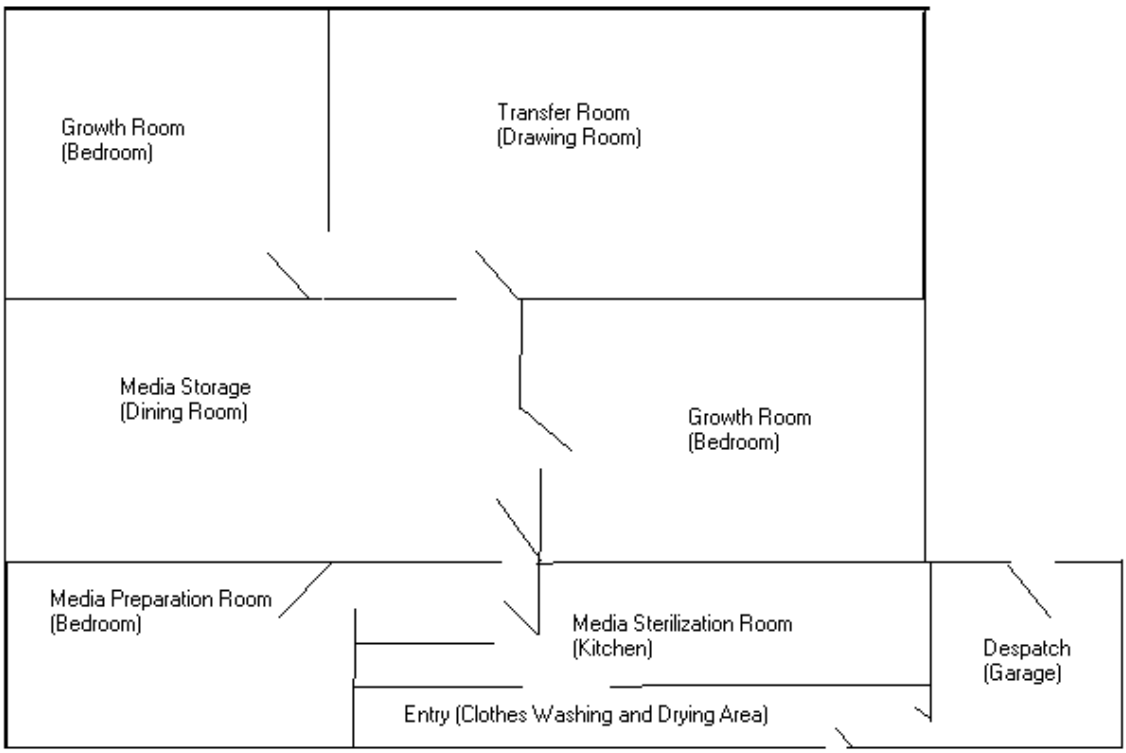

Figure 1. Conversion of a house into a micropropagation facility.

STRATEGIES FOR COST REDUCTION

For a commercial tissue culture unit to be successful, it is essential to constantly find means to increase the efficiency of production, and bring down the cost of production. A number of low-cost alternatives can be used to simplify various operations and reduce costs in a tissue culture facility.

Contamination control

The loss of cultures increases the cost of production. Contamination in cultures is caused from natural contaminants such as dust, air-borne particles, bacterial and fungal spores, fibres, and hair. Man-made contamination occurs mainly from body, clothing, and from faulty procedures in the laboratory. It has been estimated that each operator generates a minimum of 1 to 5 million particles (bigger than 0.5µM diameter) per minute. Modern, high quality clean rooms have environment control systems that minimise contamination. Wearing laboratory coats (which are mandatory in Class 100 clean rooms) reduces contamination from clothing, skin and hair. Maintaining cleanliness in other working areas is equally important. There is continuous entry of contaminants with the worker and through air entering the growth room. A set routine procedure should be followed to reduce contamination in the

laboratory; however, such procedures will vary with the location (Bruderere and Luwa, 1983; Schicht, 1985). In the absence of high cost systems, careful planning and appropriate design of the laboratory goes a long way in reducing contamination.

Washing and sterilising operations

Costly dishwashing machines for cleaning the culture containers should be replaced by manual washing if labour is relatively inexpensive. The washed culture containers can be dried in sun instead of costly hot air ovens. In small-scale laboratories, the autoclaves can be replaced by large sized pressure cookers, which are much cheaper. Instead of having one or two huge horizontal autoclaves, which generate hot air pockets in the sterilising rooms, it is better to have more of smaller vertical autoclaves, which keep the air cool. It is a normal practice to use costly aluminium foil for wrapping instruments for sterilisation. This can be replaced by stainless steel containers, which are autoclavable. Savings can also be made in media and containers (Cf. Chapter 4).

Transfer operations

Instead of Petri dishes as stage for manipulation of culture, stainless steel plates, ceramic tiles and brown wrapping paper can be used; all of these can be autoclaved. Ethanol for hand and workbench sterilization can be replaced by industrial spirit. The careless handling of inflammables used for sterilization can be hazardous. Glass bead sterilizers can be used to sterilize forceps and scalpels instead of the conventional flame sterilization using spirit lamps or gas cylinders. Commercial bleach has also been successfully used in several laboratories for bench and instrument sterilization to reduce cost, and to prevent fire hazard.

Culture maintenance in growth rooms

Plant cultures can be maintained in rooms with air conditioners and tube lights instead of highly priced plant growth chambers. The conventional method of downward illumination can be replaced by sidewise lighting systems, which not only reduces the number of lights but also provides more uniform illumination to the cultures. In the tropical and Mediterranean regions, the electrical lighting systems can be replaced by sunlight (Cf. Chapter 5). The tissue culture laboratories in Cuba produce millions of tissue-cultured sugarcane, pineapple and banana plants using natural light.

Production planning

Most plants are seasonal in demand. To meet the requirements of extremely large number of plants, commercial production has to be backed by well defined working procedures and monitoring performance of the operation. Commercial laboratories should produce a range of plants for different seasons to maximise the use of the facilities throughout the year. This lowers the unit cost of plant production. Large-scale micropropagation is a labour-intensive process, and therefore organising the availability of personnel is quite important. To reduce organisational problems in big units, the management structure must be well planned. Supporting information systems such as inventory control, production scheduling, space utilisation and daily targets should be well defined. To produce quality products on large-scale, there should be good co-ordination among the technicians, supporting staff, supervisors and the researchers. The job description and reporting system should be very clearly stated. In addition, personnel selection and training is critical for successful large-scale production. The production needs to be periodically reviewed to meet needs of the customers.

Scale of operation

The physical components required for the functioning of a commercial tissue culture laboratory can be either scaled up or down according to the interests of the propagator. The correct design of a laboratory, big or small, will help maintain asepsis, thereby increasing the efficiency of the unit and achieving a high standard of work. A micropropagation company in India converted a three-room apartment into a medium-sized tissue culture laboratory. Plant such as *Spathiphyllum*, *Syngonium*, *Ficu*s, hosta, calla lily, gerbera, and cordyline, were produced on a commercial scale. Delivery of the tissue-cultured plants to the tune of 2.5 million per year has been made from this unit by adopting various low cost alternatives. Similar tissue culture facilities, aptly called 'bio-factories', in villages of Cuba produce up to one million banana plants and 2.5 million sugarcane plants annually.

Significant cost reduction for large-scale production can be achieved with automation and mechanisation. Although, automation of certain steps of micropropagation has been investigated for the past 20 years, its commercial use has not been adopted. The capital costs of such automated systems have prevented their application.

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CULTURE MEDIA AND CONTAINERS

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Abstract. The composition of culture media used for shoot proliferation and rooting has a tremendous influence on production costs. The type of culture vessel influences the efficiency of transfer during subculture and production of propagules per unit area. Proper choice of media and containers can reduce the cost of micropropagation. The replacement of expensive imported vessels with reusable glass jars and lids, alternatives to gelling agents, use of household sucrose, and some medium components can reduce costs of production Bulk making of media and storage as deep frozen stocks also reduces labour costs.

INTRODUCTION

In vitro growth of plants is largely determined by the composition of the culture medium. The main components of most plant tissue culture media are mineral salts and sugar as carbon source and water. Other components may include organic supplements, growth regulators, a gelling agent, (Gamborg *et al*., 1968; Gamborg and Phillips, 1995). Although, the amounts of the various ingredients in the medium vary for different stages of culture and plant species, the basic MS (Murashige and Skoog, 1962) and LS (Linsmaier and Skoog, 1965) are the most widely used media.

During the past decades, many types of media have been developed for *in vitro* plant culture (Street and Shillito, 1977; Pierik, 1989; Torres, 1989). Media compositions have been formulated for the specific plants and tissues (Nitsch and Nitsch, 1969; Conger, 1981). Some tissues respond much better on solid media while others on liquid media. In general, the choice of medium is dictated by the purpose and the plant species or variety to be cultured. The ratio of auxins to cytokinins in the culture medium is important since their combination determines the morphogenic response for root and shoot formation. Plant extracts such as coconut milk, banana extract, and tomato juice can be very effective in providing undefined mixture of organic nutrients and growth factors. A variety of other media components have also been used for specific purposes. The osmolarity of the culture medium, agitation, and aeration of suspension cultures have an important influence on plant cell division. The medium can be solid, semi-solid or liquid, depending on the presence or absence of gelling agents.

LOW COST MEDIA OPTIONS

Media chemicals cost less than 15% of micro-plant production (Prakash, 1993). In some cases the cost may be as low as 5%. Of the medium components, the gelling agents such as agar contribute 70% of the costs. Other ingredients in the media - salts, sugar and growth regulators - have minimal influence on production cost and are reasonably cheap. However, low cost options are available to replace expensive gelling agents, sugars and reduce the cost of water. For example, the water from distillation apparatus or passing through Millipore filters can be replaced with ordinary tap water in some cases.

Gelling agents

The growth of cultures and production of shoots or roots is strongly influenced by the physical consistency of the culture medium. Gelling agents are usually added to the culture medium to increase its viscosity as a result of which plant tissues and organs remain above the surface of the nutrient medium. Many gelling agents are used for plant culture media, e.g., agar, 'Agarose', and 'Gellan gum', and are marketed under trade names such as 'Phytagel, Gelrite' (Sigma Co., Merck & Co. Inc, Kelco division), and 'Gel-Gro' (ICA Biochemicals). Agar is the most commonly used gelling agent for preparation of solid and semi-solid media. It contributes to the matrix potential, the humidity and affects the availability of water and dissolved substances in the culture containers (Debergh, 1983). Various brands and grades of agar are available commercially, which differ in the amounts of impurities, and gelling capacity. Agar brands vary widely in price, performance and composition. It is the actual use and experience, which ultimately determines the choice of agar brand in a specific system and for a plant species. It is usually unnecessary to use high purity agar for large-scale micropropagation; cheaper brands of agar have been successfully used for industrial scale micropropagation (Boxus, 1978).

The lowest concentration of agar, which can be used, depends on its purity and brand. Agar is usually used at 0.6-0.8% (w/v). It is advisable to prepare sample media in small quantities using various concentrations of agar, e.g., 0.7, 0.75, 0.8, 0.85 and 0.9%. The appropriate concentration should then be used for large-scale production purposes. In addition to cost saving, there are a number of other advantages in using low concentrations of gelling agents. A semi-solid medium ensures adequate contact between the plant tissue and the medium. It is beneficial to growth as it allows better diffusion of medium constituents, and is easily removed from plantlets before their transfer to *in vivo* conditions. For these reasons, a semi-solid medium is often preferred over solid medium.

Alternatives to agar

Cheaper alternatives to agar include various types of starches and plant gums (Pierik, 1989, Nagamori and Kobayashi, 2001). The National Research Development Corporation, India (NRDC, 2002) has listed low cost agar alternatives, which are worth evaluating for routine use in commercial micropropagation. Gelrite can be replaced with starch-Gelrite mixture (Kodym and Zapata, 2001). The use of liquid media eliminates the need of agar. Other options include white flour, laundry starch, semolina, potato starch, rice powder and sago. For micropropagation of ginger and turmeric, the combination of certain gelling agents gave growth as good as on agar-based media (Table 1). The use of laundry starch, potato starch and semolina in a ratio of 2:1:1 reduced the cost of gelling agent by 70-82% (Prakash, 1993). However, the addition of such gelling agents to the medium also has some disadvantages. Some gelling agents contain inhibitory substances that hinder morphogenesis

(Powell and Uhrig, 1987), and reduce the growth rate of cultures. Moreover, toxic exudates from the cultured explants may take a longer time to diffuse. Media solidified with gelling agents increase the time to clean the culture containers. The low cost options to agar, agarose, and gellan gum are listed below (Table 1 and 2).

Corn-starch (CS) as a gelling agent has been used along with low concentration of 'Gelrite' (0.5 g 'Gelrite' + 50.0 g CS /l) for the propagation of fruit trees, such as apple, pear and raspberry, banana, and sugarcane, ginger and turmeric (Zimmerman, 1995; Stanley, 1995). The shoot proliferation was better on corn starch-medium than on agar. The cost of CS was $1.8/kg compared with $200/kg of agar. However, it became difficult to detect the contamination because the CS medium turned grayish-white. Addition of 8.0% tapioca starch to the MS medium was found to be a good substitute for 'Bacto-agar' for potato shoot-culture (Getrudis and Wattimena, 1994). Barley starch (60.0 g/l) has also been used for culturing potato-tuber discs, and for anther culture of barley (Sorvari, 1986; Sorvari and Schieder, 1987). Sago (obtained from the stem pith of *Metroxylon*) at 13% concentration was substituted for agar in MS medium for the multiplication of chrysanthemum. The number of shoots and leaves, and root length were significantly higher on sago than on agar (Bhattacharya *et al*., 1994). The cost of sago is $0.5/kg. 'Isubgol', a colloidal mucilaginous husk (chiefly composed of pentosans) derived from the seeds of *Plantago ovata*), has a good gelling activity, and has reasonable clarity in gelled form. 'Isubgol' at 3% in MS medium has been used for the propagation of chrysanthemum (Babbar and Jain, 1998; Bhattacharya *et al*., 1994). The cost of 'Isubgol' is about $4/kg.

Table 1. Effect of agar alternatives on medium solidification and culture growth

Agar alternative	Effect on medium and culture
Wheat flour (10%)	Sloppy medium, growth of cultures poor
Wheat flour (8%)	Proper solidification, growth of cultures below average
Laundry starch (6%)	Proper solidification, growth of cultures average
Semolina (5%)	Sloppy medium, growth of cultures average
Potato powder (7%)	Sloppy medium, growth of cultures average
Rice powder (11%)	Sloppy medium, growth of cultures poor
Sago (7%)	Proper solidification, growth of cultures normal
Laundry starch + Potato powder + Semolina (2:1:1)	Solidification, growth of cultures as good as on agar media

(After Prakash, J. 1993.)

Use of liquid media and physical matrices

Suspension cultures without gelling agents are commonly used for culturing callus, cell clusters, buds and somatic embryos. Suspension systems allow greater contact between the explant and the medium. Moreover, agitation of such media reduces the diffusion gradient in the nutrient supply. The toxic metabolites exuding from the tissues are also dispersed effectively.

Sterilized, non-chlorine bleached, rolled, pure cotton fiber has been successfully used for culturing callus and proliferating shoots of *Taxus*, *Agrotis* and *Artemisia* (Moraes-Cerderia *et al*., 1995). Recently, the use of cotton fiber support to cultures in liquid media has been reported in the commercial propagation of orchids, banana, chrysanthemum, and potato in Pakistan and Bangladesh. Cotton fiber and MS liquid medium produced rapid growth of banana shoots, which could be sub-cultured after two instead of six weeks (S. Khan, *personal communication*). Protocorm initiation and shoot development of orchids was much faster on cotton than on agar-based medium. The cost of cotton fiber is about $2/kg, and of agar $100-200/kg depending on the manufacturer). Other alternative culture supports include foam-plastic, filter paper bridges, glass beads (MacLeod and Nowak, 1990), 'Viscose' sponge, glass wool and rock wool in liquid media.

Table 2. Low cost matrices

Option	Use	Reference
Cotton fiber	Callus maintenance and shoot organogenesis	Moraes-Cerdeira *et al*., 1995; Khan, Personal communication
Glass wool	Multiplication of chrysanthenum	Bhattacharya *et al*., 1994
Nylon cloth	Multiplication of chrysanthenum	Bhattacharya *et al*., 1994
Polystyrene foam	Multiplication of chrysanthenum	Bhattacharya *et al*., 1994
Glass beads	Multiplication of raspberry and white clover	McCulloch *et al*., 1994; T. Brinks, Univ. Hannover; Personal communication
Filter paper	Multiplication of chrysanthenum, potato	Bhattacharya *et al*., 1994

Strips (2.5x15 cm) of glass wool, nylon and filter paper have been used as supporting bridges for the propagation of chrysanthemum on MS medium. The shoot- and root-growth was almost identical on these matrices (Bhattacharya *et al*., 1994). Polystyrene foam blocks (2x2x1 cm) have been used as supporting matrix for the propagation of chrysanthemum (Bhattacharya *et al*., 1994). The growth response was similar to that on glass wool, except that explants on foam produced significantly fewer roots but longer shoots than on glass wool.

Glass bead based liquid-media were successfully used for culturing ginger and turmeric, and on per plant basis reduced the medium cost by 94% With glass beads, the amount of medium required is only 15-18 ml per culture container. By using 20ml medium per culture container, one liter media will give 50 culture containers, a substantial saving in medium cost. Ginger and turmeric plants multiplied on glass bead liquid-medium performed as good as on agar-based medium. A similar type of response was observed for vanilla. *Ficus* cv.'mini lucii' showed higher multiplication rate although with a slight vitrification. *Saintpaulia*, *Syngonium*, *Philodendron* and *Spathiphyllum* also showed higher multiplication rates and better growth on glass bead liquid-medium (Prakash, 1993). An additional advantage of liquid media with

glass beads is that the medium can be replaced without moving plants (MacLeod and Nowak, 1990).

Glass beads have been used for the propagation of raspberry and white clover resulting in 60% saving in media cost. The glass beads allowed easy removal of plantlets from the medium. Glass beads have been also used for the maintenance of callus and shoot organogenesis (McCulloch *et al*., 1994; T. Brinks, *Personal communication*). The beads can be reused after washing with acid. The liquid-media have some disadvantages. Delicate tissues get damaged during agitation. In some species, shoots submerged in liquid media become hyperhydric (water soaked), and unsuitable for micropropagation.

Conventional carbon sources and low cost alternatives

Sucrose is the most commonly used carbon source in the micropropagation of plants. Sucrose adds significantly to the media cost. Household sugar and other sugar sources can be used to reduce the cost of the medium. Sugar sold in grocery stores is sufficiently pure for micropropagation. For culturing ginger and turmeric, all other carbohydrates except sugarcane juice, were suitable alternatives to laboratory grade sucrose (Table 3). Use of common sugar reduces the cost of the medium between 78 to 87%. The cost of the local sugar was US$ 0.55/kg against the $40.0/kg for the imported sucrose. In Bangladesh, several laboratories have used locally available household sugar for culturing potato, banana, orchids, chrysanthemum, lentil, peanut, chickpea, medicinal plants, and fruit trees. In Pakistan, local sugar was found to be as good as the high grade laboratory sugar for the multiplication of banana (S. Khan, *Personal communication*). Maple syrup (from *Acer saccharum)* has been used for the multiplication (50 g/l) and rooting (34 g/l) of cherry root stocks from nodal segment and shoot tips (T. Brinks, *Personal communication*).

Table 3. Effect of alternatives to analytical grade sucrose on culture growth

Alternative	Effect
Household sugar (3%)	Healthy cultures
Double refined sugar (3%)	Healthy cultures
Sugar crystals (3%)	Healthy cultures
Sugarcane juice (10% v/v)	Drying of leaf tips
Sucrose LR grade (3%)	Healthy cultures

Adapted from Prakash, 1993.

Alternatives to other ingredients

Several other ingredients can also be replaced by low cost options Commercial grade chemicals of lower purity than the analytical grades are quite suitable for commercial micropropagation unless deleterious effects are observed. A high degree of purity is justified only in the case of basic studies in tissue culture. In general commercial micropropagation, the quality will hardly be affected ordinarily by purity of these chemicals. Of all the chemicals, growth regulators (hormones) are the most expensive. However, they are needed in very small amounts in the medium, thus have a little effect on the medium cost. Sugar cane molasses can provide many of the nutrients,namely, sugar, vitamins and inorganic metal ions required for sugarcane callus induction and shoot formation (Dhamankar,1992).

At Dhaka University, low cost media have been developed for the multiplication of orchids (Hossain, 1995; Pervin, 1997; Komol, 1998; Hoque *et al*., 1994, 1999; Zahed, 2000) based on macro-salts of any of the Vacin and Went, Phytamax and Knudson's C media, supplemented with 10-15 % (w/v) banana extract and 10-15 % (v/v) coconut water. Use of above media eliminated the need of micro-salts and vitamins for the micropropagation of different orchid species. Several different orchids, namely, *Vanda*, *Dendrobium*, *Aerides*, *Acampe* and *Spathoglottis* can be multiplied on a medium containing only peptone, inositol, banana extract and coconut water (Pervin 1997). In many tropical orchid species, similar response was observed on media containing coconut water (15%) and banana pulp (100 g/l), which effectively lowerd the cost.

Table 4. Low cost option for sugar in medium

Sugar type	Use	Reference
Refined white sugar (RWS)	Culture of zygotic embryos	Bonaobra *et al*., 1994; Okuno *et al*., 1996; Sorvari, 1986
Unrefined light brown sugar	Culture of zygotic embryos	Bonaobra *et al*., 1994
Unrefined brown sugar,	Culture of zygotic embryos	Bonaobra *et al*., 1994
Sugar maple syrup	Multiplication and rooting of cherry rootstock. Replace micro-nutrients and reduce macro-nutrients	T. Brinks, Univ. Hannover, Personal communication
Table Sugar	Multiplication of banana, potato, orchids, chrysanthemum; shoot regeneration and rooting of lentil, peanut, chickpea	Kodym and Zapata, 2001

Source of water

Water is the main component of all plant tissue culture media. Usually in tissue culture research, distilled or doubled distilled and de-ionized water is used. Distilled water produced through electrical distillation is expensive. In some cases, alternative water sources can be used to lower the cost of the medium. If tap water is free from heavy metals and contaminants, it can be substituted for distilled water. Tap water has been used for *in vitro* propagation of banana (Ganapathi *et al*., 1995) and ginger, *Zingiber officinale* (Sharma and Singh, 1995). Table bottled water from the supermarket can also be used a low cost alternative. However, its mineral composition should be taken into account as it may affect pH and nutrient uptake (H.J. Jacobsen, University of Hannover, *Personal communication*). In rural areas, rainwater can be collected in clean glass jars and used for tissue culture. In Bangladesh, the change over of water distillation from electrical to gas operated unit reduced the cost from US$260 to $5/month for producing 50-60 liter water per day (A. Razzaque, BRAC Biotech, *Personal communication*).

Low cost option for media making

Media preparation is a time consuming process. Many companies sell ready-made media in liquid or powder form. The pros and cons of laboratory making verses buying ready-made media should be considered. Although pre-made media save time, their relative cost is high. Pre-packaged media preparations are useful when medium quantities required are small. The errors in media preparation from pre-packed media are also less frequent. However, for large-scale use, it is much more economical to prepare media by mixing the basic ingredients. By making large batches, the time for media preparation and sterilization can be reduced substantially. However, if mistakes occur, the bigger is the batch, the greater is the loss (De Standaert, 1991).

The volume of medium prepared in large sized-laboratories can range from 200 to 500 liters per day. The media should be made and used in small batches. Non-sterile media should not be stored at room temperature for more than 24 hr, especially when it contains high amounts of sucrose. These should be refrigerated until further use. Media are usually dispensed into containers either manually or with a peristaltic pump. Manually, one person can fill more than 200 containers an hour. In some large-scale laboratories, the distribution of sterile media is automated. To be economical, the automation must be able handle many hundred containers per hour.

Handling stock solutions

Usually the stock solutions are prepared in 10x to 1000x concentrations. The stock solutions consist of groups of chemicals, e.g. macronutrients, micronutrients, vitamins and plant growth hormones. The inorganic chemicals and vitamins solutions can be combined into a single, 10 X concentrated, stock solution. The stock solutions can then be frozen in plastic bags in volumes sufficient to make 1 to 5 liter media at a time. The cost of the medium prepared in the above way is much cheaper than the ready-made powdered media. This is being practiced for long time in many developing countries.

Cost of medium

Depending on the plant species, the cost of culture medium varies with the ingredients such as sugars, gelling agents, and growth hormones used. Based on the current price of the various ingredients, the estimated cost of one-liter MS medium works out to be $0.18 for the solid and $0.08 for the liquid medium. If sucrose were not added, as in the case of autotrophic micropropagation, cost per liter would be $0.13 for the solid and only $0.03 for the liquid medium. The cost of medium per plant depends on the amount dispensed and the rate of plant multiplication per culture container.

Autotrophic micropropagation

Plants with functional chloroplasts can grow *in vitro* on media without sugar, provided the micropropagation environment is modified to enable photosynthesis. The growth of plants on sugar-free medium, but with the carbon dioxide enriched environment was similar to sugar-containing media (Kozai and Iwanami, 1988). Plants such as carnation, chrysanthemum, *Cymbidium*, *Primula*, potato and strawberry have been successfully grown using the above technique, termed as PTCS-'Photoautotrophic Tissue Culture System'. In the

autotrophic system, plants are grown in large containers where the air content (oxygen, carbon dioxide, relative humidity, etc) and the composition of the culture medium is easily controlled (Fujiwara *et al*., 1987). This system has several advantages that include reduction in production cost because of large culture vessels, simple culture media formulations, and lower incidence of culture contamination. Plants grown in such strongly aerated vessels require little or no hardening.

CULTURE CONTAINERS

A wide variety of culture containers are available on the market. The plant performance and cost of the containers should be used as the prime criterion in choosing the appropriate type. Depending upon the scale of production, different types of containers are deployed for culture initiation, maintenance of mother cultures and sub-culture for multiplication. For shipment of plants, disposable containers should be bought as and when required. Irrespective of the type, the containers used for maintaining *in vitro* plants should be transparent to facilitate illumination and easy inspection.

Types of culture containers

Glass test tubes have been universally used for culturing plant tissues as their narrow openings keep out contamination. Usually, only 1 to 2 explants are cultured in each test tube. As explant growth and multiplication progress, the cultures are transferred to larger containers. Test tubes should not be used for large-scale multiplication of plant material. Conical flasks, 150-250 ml capacity, are mostly used for the micropropagation of plants in liquid media, and are kept on shakers for agitation of the medium and suspension cultures. However, conical flasks are more expensive than glass bottles, and their narrow mouths make manipulations of cultures difficult. Glass and plastic Petri dishes are also used to culture explants, which are then transferred to larger vessels for multiplication and elongation. Glass Petri dishes have to be sterilized before pouring medium that has to be sterilized separately. Glass Petri dishes are more expensive than the test tubes and conical flasks. Pre-sterilized disposable-plastic Petri dishes are much cheaper. In both cases, medium is poured under the laminar flow hood.

Glass bottles and baby-food jars with polypropylene caps are the most widely used containers and the most economic and low cost option. The wide mouth makes culture manipulation easy and approximately 15-20 explants can be inoculated in each bottle. Such containers are widely available and cost ca. US$0.09. Such containers can be washed, sterilized and reused repeatedly. In Cuba, glass containers are washed with a circular nylon brush mounted on an electric rotor, and then dried in the sun by inverting them on wooden trays. The containers are then double wrapped in newspapers and sterilized in an autoclave. The medium is sterilized separately in flasks, and then dispensed into the containers under the laminar flow cabinets.

Transparent plastic containers, such as 'Magenta'^{TM} vessels, that withstand autoclaving and washing, are extensively used for micropropagation in many developed countries, but at US$1.5 to 2.0 are very expensive. Also, the repeated autoclaving of the plastic containers renders them cloudy, thus reducing the passage of light. Lately, containers are being made of polypropylene and polycarbonate and more recently polystyrene that can be autoclaved.

Disposable, non-autoclavable food containers and sandwich boxes made from polystyrene have been also used for plant micropropagation. Use of disposable containers

eliminates the cost of washing. PVC pots and jars, manufactured for the food-industry, have been used for *in vitro* plant culture. The high temperature during their manufacture imparts relatively high degree of sterility, so that they can be used directly without sterilization. Hence, only the medium needs to be sterilized, thus eliminating the costs of container sterilization. Non-sterile disposable plastic type containers and bags can be bulk sterilized with gamma rays at industrial irradiation facilities. Such gamma-irradiated containers are being used for large-scale micropropagation. Being lightweight material, these can be shipped to distant places at a very low cost.

A new type of culture vessel called 'StarPac' ^{TM} has been manufactured (Agristar Inc., Sealy, Texas). It is a disposable bag, and is semi-permeable to gas but requires different methods of handling in contrast to test tubes and other containers. Disposable plastic type containers called 'Watson Modules'^{TM} (Navrish Ltd., Arkley, Barnett, Herts. U.K.), have been used for production of micro- and mini-tubers of potato (Ahloowalia, 1999a, b). The modules allow *in vitro* multiplication, plant hardening and soil growing in the same container, and permit batch handling of cultures during all stages of plant growth (Ahloowalia, 1995). Disposable containers have been also made from fluorocarbon plastic films (Neoflon polymer film, Daikin Industries Ltd., Osaka, Japan), which can be autoclaved, and are transparent and impervious to water, but fairly permeable to gases (Tanaka *et al*., 1988; 1991).

Vessel closures and lids

The vessel closures influence growth of the *in vitro* plants. The culture containers have to be closed to keep out microorganisms but they should not be sealed so tightly that gaseous exchange is prevented. The different types of closures include non-absorbent cotton plugs, Polyurethane foam plugs, specially made plastic plugs, aluminum foil, stainless steel caps, Polypropylene caps with bends, PVC film, Polythene film and silicon rubber. Culture containers such as tubes and flasks are closed with non-absorbent cotton wool, sometimes wrapped in muslin gauze. But under large-scale production, preparing cotton plugs becomes cumbersome and time consuming. Hence, these have been replaced with autoclavable screw caps made of stainless steel or polypropylene. Test tubes covered with translucent caps, which facilitate good gaseous exchange, have been used for culturing carnation (Kozai and Iwanami, 1988). Polypropylene film, which is heat resistant, has been successfully used as closures (Crisp and Walkey, 1974; Mahlberg *et al*., 1980). It is transparent, non-greasy, and can be used to seal large containers. Sandwich wrapping films, such as 'Cling film' has also been used either on its own or as double wrap on top of the lids to prevent contamination after subculture. This is particularly suitable for mother culture, which may have to be maintained for 3 to 6 months or longer.

Gaseous exchange is important for the availability of oxygen and carbon dioxide, water vapour and elimination of ethylene gas build-up that is detrimental to plant growth. The shape of the container influences the growth rate of cultures by modifying gaseous diffusion (Bateson *et al*., 1987). The best size and shape of the culture container varies with the plant species, and hence should be decided after prior experimentation. It is important to ascertain the price, costs for washing, and the number of times the culture container can be reused. The selection of the containers should be done after making sure that they would not affect growth *in vitro*. Culture containers can be vented by modifying lids. to facilitate optimal growth. Depending on the plant species, microporous membranes of different sizes are available (Osmotec, 2002).

Low cost options for containers

Plastic bags (approximately 10x15cm) have been used for large-scale micropropagation and are very cost effective. Disposable plastic bags eliminate the cost of washing and of lids. Plastic bags are sterile due to high temperature during manufacture. After pouring pre-sterilized medium under the laminar flow, the top 2 to 5 cm of the bags is folded and several bags are held together with either large paper clips or plastic cloth-hanging pegs. After transfer of the explants and cuttings, the bags are closed with a heat-sealing machine or by knotting if the bags are 18-20 cm long. Plastic bags being lightweight can also be hung by thread and do not need elaborate shelving.

In Bagladesh and India, juice, jam, and jelly bottles have been used. The orchid producers in Thailand, Singapore, Indonesia, Malaysia, and India have been using old whisky bottles for orchid culture for many years.

The production cost of tissue-cultured plants can be reduced by 50-90% by using low cost media-ingredients and containers described in the present chapter. A private company in India has been producing more than five million plants of various ornamentals (foliage and flowering), plantation, medicinal, farm and forestry plants using low cost media ingredients and containers.

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LOW COST OPTIONS FOR ENERGY AND LABOUR

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Abstract. Artificial lighting of cultures in the growth rooms is one of the most expensive and inefficient methods in tissue culture technology. Changing illumination from artificial to natural light is a decisive low cost option in tissue culture. This reduces electricity and capital costs and also improves plant quality. Maintaining *in vitro* cultures at a regulated temperature with air conditioners adds to the cost. Many *in vitro* growing plants can tolerate wide fluctuations in temperature, and adapt better to field conditions than those grown under even temperature. Plants hardened under natural light are sturdy, and withstand transplantation better in the field. Production of plants based on tissue culture technology and their subsequent growing is a labour intensive system. Even in developing countries, where labour is relatively less costly, increasing the efficiency of production is relevant to reduce the cost of tissue-cultured plants Hiring expertise from established R&D laboratories reduces overhead costs and is indispensable to reduce losses from contamination.

INTRODUCTION

Reduction in the cost of energy and labour is essential to lower the production cost of micropropagated plants. A large part of the electrical energy in tissue culture is used for autoclaving, lighting of the growth room, air filtration in laminar-flow cabinets and air conditioning. In developing countries, the cost of electricity can account for up to 60% of the production costs. Moreover, its erratic supply and voltage create major problems. Whereas the need for electrical energy is absolutely essential, adopting low cost options can a major saving.

LOW COST ENERGY OPTIONS

Use of natural light

Artificial lighting of cultures in the growth rooms is one of the most expensive and inefficient methods in tissue culture technology. The lights, chokes, fixtures, timer controls, equipment to handle high electrical load, and their operation and maintenance add to high costs. Moreover, artificial lighting generates heat that has to be dissipated by cooling and air-conditioning further adding to the electrical load. Although special fluorescent tubes are used to compensate for the red and far-red part of natural daylight, artificial light quality does not match that of natural light under which the plants are ultimately grown. Also, the cool fluorescent lights used for illumination provide minimal energy required for photosynthesis. As a result, *in vitro* plants adapt to low-light intensity, and have a reduced growth rate.

Plants can adapt to a wide range of conditions by changing their metabolism and structures. They develop structural and anatomical features in leaves and stems, e.g. cuticle and wax on leaves, thickness of the leaf, fewer and closed stomata, and thickening of epidermal cells of the stems, that allow survival in the harsh environment. However, once they adapt to a set of conditions, re-adaptation to new conditions is rather slow or difficult. Plant tissues formed and adapted to low light conditions are usually fragile and may become

vitrified, leading to poor survival under field conditions. This can be a major disadvantage in the plant hardening process, and later establishment in the field (Cf. Chapter 9).

Under artificial light of low intensity, plants have low reserves, and a poor root system. On transfer to soil, the *in vitro* formed roots have to adjust to soil solutes of varying pH. The usual response of the *in vitro* formed roots is that they stop functioning in soil and new roots are formed, which take over the function of the original roots. If new roots do not emerge, the plant dies. One method to circumvent these negative effects is to culture the *in vitro* plants under natural light, during their last phase in liquid medium, based on half- or quarter-strength MS salts without sugar and vitamins, under either aseptic or non-aseptic conditions. If roots or root initials are not formed, the medium can be either supplemented with auxins (IAA, IBA), or shoots dipped in a solution of rooting hormones. This procedure provides much stronger and healthier plants with a high survival rate.

Low cost lighting

Changing the method of illumination from artificial to natural light is a decisive low cost option in tissue culture. This not only reduces electricity and capital costs, but also improves the plant quality. Expensive artificial lights can be replaced in several ways. One option is to grow the *in vitro* cultures in diffused natural light under plastic or glass. This works very well in temperate climates, but under tropical conditions, heat build-up has to be reduced by installing thermostat-controlled exhaust fans. It is also possible to redesign the existing laboratories in tropical and subtropical countries, and replace the artificial lights with 'Solatube' (Anonymous, 2001), which can be installed in any roof, and redirects the daylight through reflective tubing (Kodym and Zapata-Arias, 1999, 2001; Kodym *et al*., 2001). Such a tube currently costs ca. US $600, and once installed can illuminate 3-5m^2 without incurring further running costs. Yet another method is to incorporate Southwest facing glass windows in the growth rooms that allow indirect diffused natural daylight. By using either muslin or Venetian curtains made of bamboo or plastic, the light can be diffused to the desired intensity. The growth room is usually located on the upper storey of the house to allow sunlight from all directions. Natural light has been successfully used in this manner in "Bio-factories" in Cuba, based on the conversion of village houses into tissue culture laboratories (Baezas-Lopez, 1995).

In vitro cultures have been also successfully grown under natural light by using simple plastic bags (polypropylene bags) as culture containers. These bags are lightweight and can be hung in a greenhouse. They are transparent, so that incident illumination reaches the cultures with the least loss, and near natural spectrum. The plants undergo primary hardening while they are still developing in the culture. Such plants have well-developed stems, leaves, and good growth vigour, and survive better on transfer to soil. With proper precautions, contamination of cultures in the bags under greenhouse conditions can be avoided. This system of culturing plants under plastic and greenhouse also eliminates the need, and hence the cost, of air filtration and air conditioning commonly used in the sophisticated facilities. Artificial lights in other laboratory areas such as media preparation, transfer and hardening rooms can also be minimized by incorporating in the room design glass windows that provide indirect sunlight but reduce heat build-up in the rooms. This can be achieved by using either muslin net curtains or translucent glass.

Low cost temperature regulation

Maintaining *in vitro* cultures at a regulated temperature with air conditioners adds to the cost but does not contribute to specific plant quality. In fact, as in the case of artificial lighting, plants grown under a narrow temperature range are at a disadvantage during hardening and later under the field conditions. Elimination of this factor significantly contributes to reduction in electrical costs. Contrary to the common belief that the day- and night-temperature in the growth room must be strictly controlled at an even level, many *in vitro* growing plants can tolerate wide fluctuations in temperature. High daytime temperatures of up to 28-30^{o}C and as low as 10-12^{o}C at night do not damage plant growth. On the contrary, fluctuations in temperature promote better growth. *In vitro* growing plants of banana and potato, kept at 16-41^{o}C at 750 µmol m^{-2} s^{-1} under natural light, showed as good or better growth than in the controlled growth room (Kodym *et al*., 2001).

Reducing energy costs for water and autoclaving

Normally, distilled water is produced from water stills operated by electricity. Some water stills and autoclaving require a three-phase connection. For small facilities, it is prudent to operate the units on a single-phase electrical connection. In small units, tap water may be used after autoclaving rather than distillation. Pressure cookers heated with gas can also be used where capital is a constraint. In case of large-sized facilities, autoclaves and water distillation equipment operated on electricity is still the most economic (Cf. Chapter 4).

Reducing energy in the glasshouse and hardening area

Electrical usage can be reduced significantly from hardening plants in open shade if glasshouses are not available. *In vitro* grown plants can be hardened by covering them with net-shades after transfer to soil or by placing containers in shade under thatched or plastic-covered open huts. The plants hardened under natural light are sturdy, and withstand transplantation better in the field. In the case of culture in plastic containers, the lids are removed, and containers are covered with a thin plastic sheet, and left for 3-4 days in shade. The medium is then removed by washing with tap water, and the plants are retained for another 3-5 days in the shade before transfer to soil (Cf. Chapter 9).

REDUCING LABOUR COSTS

Production of plants based on tissue culture technology and their subsequent growing is a labour intensive system. In developed countries, labour is a major cost factor in micropropagation. On the other hand, in developing countries, labour is relatively less costly, which is a major advantage. Yet, in developing countries, increasing the efficiency of production is relevant to reduce the cost of tissue-cultured plants. In developing countries, the access to capital is not easy and borrowing is costly with high interest rates, which adds to cost of production. Hence, it is important to maintain high labour efficiency, especially when the operators in the culture transfer room are not highly trained. The typical profile of a tissue culture plant production system shows that 40% costs are for labour, 10% for materials, 20% for overheads, and 30% for sales, general and administrative activities (Walker, 1986). The manual transfer of explants in culture vessels has the upper ceiling of production per operator at about 5000 operations or transfers per day. However, this peak rate is rarely achieved. In general, 2500 transfers per operator per day are considered to be good productivity, which is strongly influenced by the type of culture vessel used (Walker, 1986). For example, the

transfers per person using test tubes are much lower than that in Petri dishes and wide mouth culture vessels.

Once the efficiency of labour in transferring number of propagules per hour has been achieved to the maximum possible, there is little scope to improve efficiency unless automated or semi-automated systems are introduced. These are based on the use of liquid media and bioreactors, and mechanization to handle propagules. Such systems have been developed and can be integrated into the production under certain conditions (Osmoteck, 2000) (Cf. Chapter 6). Accordingly, such a system can reduce cost of production by 50% in a 20 million-unit production facility. The labour cost comes down from conventional to semi-automated model by 70% and in fully automated model by 75%. The system based on the use of plastic bags increased throughput per person and reduced labour cost per propagule by 60% (Savangikar, 2002). Also, the improvement in contamination control increased multiplication rate and improved survival during hardening and produced more saleable plants. In some cases, the multiplication rate increased from 1.5 to 5, reducing labour cost of tissue culture by 96% per plant before hardening. To reduce labour costs, such systems can be combined so that the multiplication phase is in the bioreactor and the last stages in plastic bags (Cf. Chapter 6).

To maintain high efficiency of the operators under laminar flow hoods, they should work not more than 4 hours per day and preferably on a single bench; afterwards they should undertake other operations, such as washing, drying of containers or work in glasshouse. The provision of music in culture transfer rooms also increases the efficiency. Usually, female operators are more efficient than males in the transfer rooms and for potting of plants. This contributes to the gender based rural employment.

Reducing overhead costs

Overhead costs for commercial scale tissue culture-based plant production include salaries of managers - directors, scientists, marketing managers, market promotional expenses, payment of commissions, and perks to the management. Much of these cannot be avoided. The large-sized companies can afford high overhead costs that match large volume of business. However, smaller units have to rely on a few permanent managers, but may hire the expertise of the specialists as and when required.

Large-scale production based on tissue culture is also prone to large-scale losses through contamination of cultures, substandard production, losses during hardening and badly planned and poor marketing. Many failures of commercial micropropagation concerns can be traced to improper planning of production and marketing. While it is important to maintain high levels of clean technology, the need for critical expertise cannot be ignored in keeping contamination to less than 5%. The high contamination rate can become a major cause of not achieving the set targets and throwing the assumed production of the enterprise out of gear and loss of market. The advice and expertise of experienced persons is indispensable to reduce such losses. The expertise can be obtained for a reasonable fee either from established R&D laboratories of the larger companies or from colleges and universities. Such laboratories can also provide services for quality control, indexing of mother cultures, advice on media formulations and trouble shooting. For sustainable technology advancement, links should be established within the private sector, and between the private and public sector R&D organizations. The shared costs of R&D in tissue culture technology by the private and public sector is thus a low cost option to the governments and policy makers in the developing countries.

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BIOREACTORS AS A LOW COST OPTION FOR TISSUE CULTURE

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Abstract. Bioreactors are vessels designed for large-scale cell, tissue or organ culture in liquid media. Functionally, plant culture bioreactors can be divided into two broad types: those in which the cultures are immersed partially or temporarily in the medium, and those in which the cultures are continuously submerged. Bioreactors provide more precise control of the plant growth gaseous exchange, illumination, medium agitation, temperature and pH than the conventional culture vessels. Bioreactor-based propagation of plants can increase rate of multiplication and growth of cultures and reduce space, energy and labour requirements in commercial micropropagation. They can therefore be attractive to developing countries as regards new or expanding plant culture facilities, in combination with a conventional laboratory. However, to be cost- effective, use of bioreactors requires indexed plant cultures, and attention to aseptic procedures during handling of plant material. Hence, the integration of bioreactors into production systems should only be attempted by facilities with skilled and experienced propagators.

BIOREACTORS

Bioreactors are vessels designed for large-scale cell, tissue or organ culture in liquid media. For an increasing number of plants, they have demonstrated a number of important advantages over conventional semi-solid micropropagation, including several fold increase in multiplication rates, and reduction in space, energy and labor. These cost-saving advantages have been the driving force for increased attention to the use of liquid systems generally. There are, however, many disadvantages because of a number of problems associated with the use of bioreactors in micropropagation. These include contamination, lack of protocols and production procedures, increased hyperhydricity, and problems of foaming, shear stress and release of growth inhibiting compounds by the cultures. Because of the inherently difficult problems of contamination in liquid systems, and the lack of problem solving information compared to semi-solid growth media, such systems involve more risk and require greater skill. Thus, in many developing countries, bioreactors have not been a low cost option. The potential of cost reduction will depend on the cost of labor, production capacity, crops being propagated, contamination rate, and energy and costs.

Bioreactor vessels

For micropropagation, “large-scale” bioreactors generally refer to vessels with volumes ranging from 1.5 to 20 l, although even larger vessels have been used (Takayama and Akita, 1994). However, even these modest volumes are in stark contrast to the standard plant tissue culture vessels, which typically contain less than 0.5l of semi-solid medium. The size of the vessels in bioreactors versus conventional micropropagation systems has important investment cost consequence. Standard semi-solid plant tissue culture vessels have a seal that permits limited gas exchange with minimal contamination. The environment of the conventional growth room determines the light, temperature and gases in the conventional vessel.

*deceased.

Some bioreactors can grow 60-100,000 plants per cubic meter and provide more precise control of the plant growth gaseous exchange, illumination, medium agitation, temperature and pH. Since they are aseptically sealed, they can be operated most of the time in ordinary rooms. Only the handling of plant material is performed under laminar flow. They can therefore be attractive to developing countries as regards new or expanding plant culture facilities, in combination with a conventional laboratory. However, to be cost- effective its use requires indexed plant cultures, and attention to aseptic procedures during the handling of plant material.

TYPES OF BIOREACTORS

Various bioreactor designs have been developed for a wide range of crops, culture types and stages. Functionally, plant culture bioreactors can be divided into two broad types: those in which the cultures are immersed partially or temporarily in the medium, and those in which the cultures are continuously submerged. The latter are usually used for high-density multiplication of cultures where submersion does not result in abnormal plant development. Such cultures include protocorm-like bodies, embryogenic callus, somatic embryos, meristem clusters and nodules. Protocorm-like bodies are meristematic tissues that regenerate propagules. Meristem clusters and nodules are organogenic culture made up of compact masses of meristems without significant leaf, stem or root development. Submerged bioreactors can also be used for the development of small bulbs or corms for field growing (Takayma, 1991).

Partial immersion type

There are several types of partial immersion bioreactors. These include gaseous phase bioreactors, liquid layer bioreactors and temporary immersion bioreactors. Such designs are preferred to culture plant material sensitive to hyperhydricity (Ziv, 1999; 2000). In gaseous phase bioreactors, the cultures are mechanically supported on a porous base and intermittently sprayed with medium (Ushiyama, 1988) or exposed to a nutrient mist (Weathers *et al*., 1988). Excess medium is collected in the vessel and re-circulated. In such bioreactors the vessels are fabricated from autoclavable clear glass or plastic, and the environment of the growth room controls illumination and temperature. These bioreactors can provide excellent growth and development for most tissue and organ cultures. However the mist generation systems available are complex to maintain.

In liquid layer bioreactors, only the base of cultures is exposed to the medium. The control of illumination, temperature and the gaseous environment is much the same as in standard tissue culture vessels. The 'LifeRaft' ^{TM}, a simple liquid layer system, consists of a disposable (or a reusable) microporous membrane raft supported by a buoyant float (reusable, autoclavable) at the surface of the liquid medium (Osmotek, 2002). Stationary support systems for liquid layer bioreactors have been also developed from sealed clear plastic film with a wire frame (Takayma, 1991). Small bags made of plastic film are also being used instead of vessels in commercial laboratories.

In temporary immersion bioreactors, the cultures are immersed in the medium, for a preset duration at specified intervals. Their construction and operation is very simple, which makes them attractive low cost alternatives. A typical design, uses two vessels (plastic or glass) of which one holds the liquid medium and the other the cultures. Another version of temporary immersion bioreactors includes a system where a single vessel with a reservoir on one side mechanically tilted at preset intervals (Adelberg and Simpson, 2002). In this manner,

medium periodically bathes the cultures in the vessel (Fig. 1) and maintains the propagules in a vertical position.

Other temporary immersion bioreactors use different designed vessels and rotation. As the vessel turns, the culture is intermittently dipped in the medium. Temporary immersion bioreactors are simple and cost effective to run. Consequently, a number of commercial micropropagation laboratories have begun to integrate these systems into their production procedures.

Fig.1. Rectangular vessels with intermittent cycle of wetting and drying on tilted shelves.

Submerged type

There are basically two types of submerged bioreactors - mechanically agitated, and air-driven. Mechanically agitated bioreactors make use of devices such as propellers, turbines, impellers, paddles, and vibratory mixers to circulate the medium. Each device creates a somewhat different pattern of fluid flow in the vessel (Preil, 1991), For each type of culture and plant, the shear force and pattern of flow must be optimized. For example, it is advantageous to have sufficient shear force to maintain meristematic clusters as small aggregates. This minimizes stagnant regions in the vessel, which can cause cultures to stick to surfaces and form clogs that disrupt medium flow. On the other hand, high shear force can produce excessive foaming, and has a negative effect on culture growth. Such a system (e.g. NLH bioreactor) for cell and embryo culture has recently been reported (Hvoslef-Eide, 2001).

Air-driven bioreactors are the simplest submerged bioreactor. In air-driven bioreactors, filter-sterilized air is forced into the vessel's bottom, and aerates and agitates the medium, thereby, lifting and circulating the culture (Merchuk, 1990). Such air-driven bioreactors are called simple aeration or bubble column bioreactors, depending on their volume. Air-driven bioreactor vessels are usually fabricated from transparent or translucent materials, but are typically formed from autoclavable clear glass or plastic and fittings. Alternatively, they are made of non-autoclavable clear flexible plastic, and sterilized with gamma radiation (Osmotek 2002). The simple airlift or bubble column bioreactors are easy and cost efficient to run. The greatest advantage of submerged bioreactors is that they can be integrated into automated or mechanized micropropagation systems (Levin *et al*., 1988; Levin *et al*., 1997). In the automated systems, culture from the bioreactor is pumped to a bioprocessor for separating, sizing and distributing the culture to vessels for propagule development. In the mechanized system, an operator transfers the multiplied culture to a mechanical cutting device for separation and then to a vessel for propagule development.

ADVANTAGES AND DISADVANTAGES

The advantages of bioreactors include increased culture multiplication rate, faster culture growth, reduction in medium cost and reduction in energy, labor and laboratory space. The increased rate of multiplication and growth primarily reflects the effect of liquid medium (Levin *et al*., 1997). The elimination of gelling agents (e.g. agar) reduces medium cost, and filter sterilization of the medium eliminates the need for autoclaving. In bioreactors, the culture density in liquid media is much higher than in the conventional vessels with semisolid media. The conventional tissue culture vessels are typically kept on shelves with a large space between the shelves. The use of bioreactors requires much smaller space in the growth room, fewer clean work stations, and less space for media preparation, vessel storage and washing than that used in the conventional micropropagation. The smaller size of the laboratory and the number of people reduce air-conditioning needs, hence energy costs. Reduced requirements for lighting the bioreactors and autoclaving, and less labor, simplification of medium preparation, washing of vessels, and simplified handling of the cultures, all lead to cost reduction.

There are, however, many disadvantages because of a number of problems associated with the use of bioreactors in micropropagation. These include contamination, lack of protocols and production procedures, increased hyperhydricity, and problems of foaming, shear stress and release of growth inhibiting compounds by the cultures. Unfortunately, culture contamination, which is a major problem in conventional commercial micropropagation (Leifert and Waites, 1990; Leifert and Woodward, 1998; Leifert, 2000), is even more acute in bioreactors. In conventional micropropagation, discarding a small number of the contaminated vessels is an acceptable loss; in bioreactors, even a single contaminated unit is a huge loss. However, despite these difficulties, a number of commercial laboratories have developed effective procedures to control contamination in bioreactors.

CONTROL OF CONTAMINATION

Prevention of contamination in bioreactors requires a proper handling of the plant material, of equipment during transfers, and cultures during production. Only the surface sterilized explants, multiplied in small vessels and indexed for freedom from diseases are used to initiate cultures in bioreactors. If the bioreactor is small, it is sterilized in an autoclavable plastic bag, sealed with a cotton wool plug, and opened only under the laminar flow cabinet. If the bioreactor is large, other methods to protect the vessel after autoclaving and during its transport to the sterile area must be provided. The vessel is assembled under laminar flow, taking care that non-sterile objects do not enter the sterile air stream in the vicinity of open ports. After inoculation, all the ports are sealed and the bioreactor is brought to the growth room and connected to an air supply. For harvesting, the bioreactor is returned to the sterile area, and the ports are opened so that the air stream cannot carry contamination from the outside. If proper procedures are followed, the reactor can be reused without re-sterilization.

Despite the precautions taken in initiating cultures, bioreactors can become contaminated from the environment or from microbes, latent in the culture (Cf. Chapter 1 and 9). The contamination can be controlled with one or a combination of anti-microbial compounds, acidification of the media, and micro-filtration of the medium. Several commercial laboratories use anti-microbial compounds, such as PPM, G-1 (2-(5-bromo-furil)-1-bromo-1-nitroethane), a fungicidal and bactericidal chemical, sodium hypochlorite or sodium dichloroisocyanurate,. Acidification of the medium (pH 3) has been used to control contamination in the multiplication of banana and in media for other crops (Leifert *et al*.,

1994). Contamination can also be controlled by circulation of the medium through a 0.2µ microporous filter (Levin *et al*., 1996). However, the removal of contaminants needs frequent filter changes, which is too expensive for use in a commercial laboratory. Chilling the filter retards filter clogging significantly.

CONSTRUCTION OF A SIMPLE BIOREACTOR

For laboratories with limited resources, a simple temporary immersion bioreactor can be constructed of autoclavable glass or plastic vessels or bags with a wide (at least 5 cm diameter) mouth and a tight fitting rubber stopper. It is better to use small vessels, of one liter or less. The stopper should have two holes for glass tubing. One tube is inserted and fixed in the stopper so that when the vessel is closed, the end of the tube is closer to the bottom of the vessel. A second tube is inserted and fixed in the stopper so that the end of this tube is near the top of the vessel when it is closed. The tubes can be fixed with a heat resistant silicone sealer, such as that used for repair of car radiators. The vessel should have at least one port (5-10cm diameter) to add or remove cultures, and the port should be sealed with a rubber stopper or a metal closure with a rubber gasket. There are four ports, two for air-inlet and -outlet, and two for medium-inlet and -outlet. The air-inlet and -outlet ports are connected to hydrophobic Teflon submicron microporous air filters, which must be kept dry. The wet filters clog and may lead to contamination. It should be possible to open and close, connect and disconnect medium ports without leakage to prevent entry of contaminants. The bioreactors should fit under the laminar flow unit leaving enough space on the top and sides to allow easy access to the vessel for handling cultures and changing the medium.

The purpose built bioreactors are either air-driven or temporary immersion type. A reservoir vessel similar to the culture vessel is prepared except that the mouth of the vessel is just wide enough to fix the two glass tubes. The tops of the tubes, that reach the base of the culture vessel, are connected to each other with silicone tubing. For both the reservoir and culture vessel, the ends of the short tubes above the stopper are connected to sterilizing filters such as ‘Acro 50’ filters (Pall Filter Corp. USA) with a short length of silicone tubing.

Prior to use, the bioreactor is assembled with the stoppers loosely fitted into the vessels and placed in an autoclavable bag with a cotton wool plug. The culture vessel can also contain an autoclavable plastic or stainless steel mesh screen to support the culture above the medium in the vessel. The bioreactor is then autoclaved and placed on the laminar flow bench. The culture is aseptically introduced into the vessel and sterile medium in the reservoir, and the stoppers are tightly closed. If necessary, the part where the stoppers fit into the vessels is taped shut to prevent contamination. The bioreactor is then placed in the growth room, the two vessels are placed alongside each other, and the reservoir air filter is connected to a 6W aquarium pump with silicone tubing. Air is pumped into the reservoir vessel, forcing the medium into the culture vessel. The medium is allowed to remain in the culture vessel for a short duration and the air pump is then connected to the culture vessel air filter, and the medium is pumped back into the reservoir. This process is repeated at preset intervals, and can be automated.A simple bubble reactor has also been described parts of which can be purchased as a kit (Ziv and Hadar, 1991; Ziv *et al*., 1998; Osmotek, 2002).

COST-BENEFIT ANALYSIS

A recent study has shown that bioreactors can significantly reduce the production cost of micropropagation in developed countries, from $0.16 to $0.07-$0.08 per unit (Gross and Levin, 1999). This has recently been confirmed in commercial laboratories. For example, a

major ornamental plant producer in the US is now propagating 40,000 units per month based on bioreactor multiplication and mechanical culture separation. The cost reduction (Tables 1-3) is based on three situations. Model 1 is a conventional laboratory producing 20 million units per year. Model 2 is a laboratory using multiplication in bioreactors, mechanical separation of the culture and elongation on semi-solid medium. Model 3 is a laboratory using multiplication in bioreactors, mechanical separation of the culture and elongation in temporary immersion bioreactors (Gross and Levin, 1999).

It is, however, unclear whether bioreactors will produce a commercially relevant cost reduction in developing countries. In countries, such as India, labor cost can be as low as US $0.20-0.60 per hour (Savangikar, 2002). Based on projections of Model 1 above, this would mean that in a developing country, a conventional laboratory with $20X10^6$ units per year capacity could produce propagules at $0.06-$0.07/unit. This projection agrees with the available information on commercial laboratories in India with 6-10 million units capacity per year, and production costs of $0.06-$0.14/unit (Barathi, 2002). For larger laboratories, bioreactor micropropagation could probably further reduce this cost by about $0.01. In addition, bioreactor micropropagation would simplify production management through the use of large culture batches and reduced labor. Although cost savings alone might not justify the integration of the bioreactors into production, the simplification of production procedures and management might make bioreactor-based micropropagation desirable for large-scale micropropagation concerns.

Table 1. Estimated annual cost (US$) of major investment for the laboratory

Building	Building Costs	Amortize Years	Annual interest %	Annual cost of building	Annual labor cost	Annual energy cost	Total Annual cost
Model 1	2696000	20	4	140190	2163600	209160	2512950
Model 2	2059000	20	4	107070	634800	1374520	879390
Model 3	1641000	20	4	85332	530400	48960	664683

Savings from Model 1-2 = 65%; from Model 1-3 = 73%

Table 2. Estimated annual cost (US$) of major equipment and furniture for the laboratory

Equipment cost	Furniture cost	Amortize years	Annual interest %	Total annual cost	
Model 1	1000000	300000	10	4	135200
Model 2	100000	30000	10	4	135200
Model 3	100000	30000	10	4	135200

Table 3. Annual cost of production (US$) for each model ($20X10^6$ units/year)

	Total Cost (A)	Total cost (B)	Costs per unit	Unit cost media	Unit cost vessels	Unit cost misc.	Total unit cost
Model 1	2512950	135200	0.13	0.01	0.01	0.01	0.16
Model2	879390	135200	0.05	0.01	0.01	0.01	0.08
Model 3	664683	135200	0.04	0.01	0.01	0.01	0.07

A =Table 1. B= Table 2.

As stated before (Cf. Chapters 4 and 5), the elimination of agar and the use of natural light in the growth room can produce a 90% reduction in resource costs (Kodym and Zapata, 2001). Similarly, the use of natural light in combination with the use of liquid media can result in over a 90% reduction in the cost production in India (Cf. Chapter 4). If the adoption of such technologies brings down the cost of production in developing countries, the cost reduction from bioreactor micropropagation would be indeed small. However, the simplification of production management could still make bioreactor micropropagation worthwhile for the large-scale laboratories. Most laboratories in India (70%) produce less than 1 million units per year (Mascarenhas, 1999). It is likely that this also reflects the situation in many other developing countries. Since the use of bioreactors will not produce a large cost reduction or greatly increase efficiency of small laboratories, the integration of bioreactors into production systems should only be attempted by facilities with skilled and experienced propagators.

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DISEASE DETECTION AND ELIMINATION

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Abstract. In micropropagation, the health status of the donor mother plant and of the plants multiplied from it are among the most critical factors, which determine the success of a tissue culture operation. The indexing of the mother plants for freedom from viral, bacterial, and fungal diseases is a normal procedure in large-scale plant propagation through tissue culture. Plants not originating from pathogen-tested material must be screened for the presence of viruses Laboratories, which do not have in-house facilities to carry out plant indexing, should obtain their indexed stock plants from organizations such as Departments of Agriculture, agricultural universities or privately owned certified germplasm repositories that routinely produce such plant material. Batches of micropropagated plants should be tested for freedom from diseases either in-house or by other laboratories. ELISA has been the most effective method for virus and pathogen detection in plants. Polymerase chain reaction (PCR) and nucleic acid hybridization are more sensitive than ELISA, and can detect pathogens in extremely low amounts. The elimination of most viruses can be achieved by a combination of apical meristem culture and thermotherapy.

INTRODUCTION

The health status of the donor mother plant and of the plants multiplied from it are among the most critical factors, which determine the success of a tissue culture operation. Hence, indexing of the mother plants for freedom from viral, bacterial, and fungal diseases is a normal procedure before undertaking propagation in large-scale plant propagation through tissue culture. Laboratories, which do not have in-house facilities to carry out plant indexing, should obtain their indexed stock plants from organizations such as Departments of Agriculture, Agricultural Universities or privately owned certified germplasm repositories that routinely produce such plant material. Batches of micropropagated plants should be tested for freedom from diseases either in-house or by other laboratories.

BACTERIAL AND FUNGAL CONTAMINATION

While most of the fungal and bacterial diseases are eliminated during surface sterilization and culture, viruses and viroids survive through successive multiplication if the mother plant is infected (Sessitsch et al., 2002). Even cultures from seeds may carry viroids and bacterial endophytes, in certain cases also viruses. Bacteria may be controlled under in vitro conditions to some extent using certain bacteriostatic agents. Nevertheless, such chemical treatments with e.g. ‘plant protection mixture’ (ppm) or commercial antibiotics even when applied on long term do not fully eliminate microorganisms.

VIRUS INFECTION IN PLANTS

Viruses are obligate parasites, and for their multiplication, they use the metabolic machinery of the cells, and inhibit plant growth. Although, many viruses do not produce disease symptoms, they adversely affect plant metabolism and increase progressively with repeated vegetative propagation (Wang and Hu, 1980). Most viruses infect only a limited

number of species, but a few have a wide host range (Mathews, 1991). Many plant viruses are transmitted through vectors such as insects, nematodes, bacteria, and fungi. Propagation from vegetative parts, grafting and sap can transmit viruses from one plant to another. The damage from viruses depends on their concentration in the tissues and spread within the plants. The greater the concentration of the virus, the greater the debilitating effect observed on plant growth. Many varieties of vegetatively propagated crops decline in performance with viral accumulation and must be discarded. Even though the viruses do not produce visible symptoms, the plants have reduced yield and poor quality. The removal of specific viruses by meristem-tip culture has been reported to lead to a dramatic yield increase (Murashige, 1980) and rejuvenation of plant varieties that are vegetatively propagated. This is a unique contribution of meristem-culture not achievable by any other techniques.

Detection of viral diseases

Plants not originating from pathogen-tested material must be screened for the presence of viruses. Procedures to detect the presence of viruses include visual examination for viral symptoms, infection tests on indicator plants, serological tests, electron microscopy, and direct detection of RNA using molecular techniques. Internationally approved and recognized detection systems include serological and molecular laboratory assays, and indicator hosts in the greenhouse.

Indexing with indicator plants

Prior to the advent of antigen-antibody reaction detection systems, the earlier method of detecting virus relied upon indicator plants. For example, in potato, viruses A, X, and Y can be detected by rubbing the juice of the infected plant on the leaves of indicator plants such as *Chenopodium spp., and Nicotiana spp.* The development of necrotic spots on the leaves is a typical symptom indicating the presence of the viruses. For example in sweet potato, its wild relative, Brazilian Morning Glory (*Ipomaea setosa*) is still used as the indicator host (Anonymous, 2002). Similarly, in many fruit trees, the appropriate rootstocks are used for indexing of various virus strains (NRSP, 1999; Arthofer, 2001). The detection of plant diseases has changed radically in the last 20 years. A number of indicator plant based tests have been replaced with specific Enzyme Linked Immuno Sorbent Assays (ELISA). Nevertheless, indexing with indicator plants is a low cost and highly effective method to detect many viruses.

ELISA test

ELISA has been the most effective method for virus and pathogen detection in plants. Each virus has a unique protein coat. Hence they can be detected by using antibodies, which are usually linked to enzymes for reporting the affinity binding via colour signal from the used enzyme substrate. The double antibody sandwich (DAS) method in a 96-well microtitre plate (Clark and Adams, 1977) is still widely used. Various alternative formats, substrates, and antibody binding modifications have been developed over the years to increase specific sensitivity (Agdia, 2002; Bioreba, 2002). The most common method nowadays uses "biotinylated" virus-specific antibodies to improve their affinity to the respective virus surface thus improving the signal. The use of monoclonal antibodies has been so far restricted to certain potato and barley viruses, because of their high cost and inability to detect the range of strains in several diseases.

Large scale testing based on screen-spot samples is also a routine procedure. Such dot-immuno-binding assays are versatile and cheaper alternatives to microtitre plate tests. Plant sap is directly spotted onto a pre-treated microporous membrane (e.g. nitro-cellulose or polyvinylidene difluoride). The test kits can be used directly in the field. Sample membranes can be dried and shipped to another laboratory for processing. However, such systems are not useful for viruses present in low concentrations in the plant sap.

ELISA has several limitations. First of all, a test is specific only to a given strain of the pathogen. For a large group of ubiquitous viruses, which do not produce disease symptoms or fatal consequences, ELISA kits have not been developed. Thus, the strain specificity has severe limitations for comprehensive quality control. Further, ELISA is less sensitive than PCR, and may fail to detect low amounts the virus in tissue-cultured plants. Whereas a positive ELISA is a good indicator of the existence of microbes (but false positives may also occur), a negative test does not ensure their absence. The use of Immuno-Tissue-Printing allows the localization of viruses in tissues and therefore the improvement of elimination strategies *in vitro* by visualizing the result of virus removal (Fitch *et al*., 2001).

Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) is more sensitive than ELISA, and can detect pathogens in extremely low amounts. PCR detects pathogens from their genetic material, i.e. DNA or RNA. This technique uses a specific enzyme and the respective genomic start code for a relevant section of the pathogen-DNA to reproduce millions of copies from it. Therefore, even minute quantities of the pathogen DNA can be detected. Since most plant viruses are made of only RNA, first a DNA copy of the RNA is made, whereas DNA from bacteria and phytoplasmas may be amplified directly. Because the genetic material of the pathogen is mixed with that of the plant, it is necessary to target only the genetic material to be copied. This is done by adding small pieces of DNA called primers which stick only to borders of the region of genetic material that need to be copied. The primers then direct the enzyme where to start and end amplification.

During PCR, the DNA is subjected to a series of hot, cold, and warm cycles in a thermocycler. Hot cycles split DNA into single strands. Cold cycles allow the primers to attach to the DNA. During warm cycles, the enzyme makes a copy of each piece of primed DNA. If a phytoplasma is present, PCR produces a large quantity of a specific piece of DNA. By using gel electrophoresis, DNA pieces are separated in size, and the specific piece of DNA is identified. If the critical concentration of a DNA particle is sufficiently low and the sample tests negative, the plant material can be regarded as practically free of the target microbe. Once a test is standardized with an appropriate primer, and by a method, which excludes inhibitors, a negative PCR test is a more reliable indicator of practical freeness. The use of PCR drastically reduces the number of samples, which test negative although they may carry the virus. Once proper probes are developed, the nylon membrane spot test is a practical, rapid and accurate method for diagnosis. PCR has the advantage that by determining "degenerate primers", a fairly large group of microbes such as viruses, bacteria and fungi can be detected.

Nucleic acid Hybridization test

The presence of viruses can be detected from isolation and characterization of double stranded RNA (ds-RNA) produced in most plants during viral replication. Although non-specific presence of ds-RNA in a sample extract strongly suggests viral infection, for further identification of the virus, the test has to be verified by serological and other methods.

Viroids are much smaller than viruses, and are made of a circular piece of RNA and do not have a coat protein. Viroids such as 'Potato Spindle Tuber Viroid' can be detected by nucleic acid hybridization. The RNA of the virus "hybridizes" with probe RNA. When the two single strands come together, they form a double strand. To detect the viroid, RNA extracted from plant tissue is attached to a microporous membrane. This bound RNA is then detected by a "probe", which is an RNA particle labeled with either ^{32}P or a chemical called DIG (digoxigenin). If a viroid is present, the probe hybridizes with it; if not, the probe is lost when the membrane is washed. DIG bioluminescence or radioactivity if present on the membrane can be detected on an exposed film. Samples from known healthy and known infected plants are used as controls. Large scale testing on nylon screen-based spot samples is also a routine procedure.

Elimination of viruses

The elimination of viruses can be achieved by a combination of apical meristem culture and thermotherapy. Meristem culture is the most commonly used method to free plants from virus diseases. This should not be confused with shoot tip culture as explained before (Cf. Chapter 1). Virus-free plants have been obtained by thermo-therapy and meristem culture in several species (Morel and Martin, 1952; Belkengren and Miller, 1962; Mullin *et al*., 1974; Boxus, 1976).

The plant meristem is a zone of cells with intense divisions, situated in the growing tip of stems and roots. The virus travels through the plant vascular system, which is absent in the meristem. Moreover the cell-to-cell movement of the viruses through plasmodesmata cannot keep up with the growth and elongation of the apical-tip. The high metabolic activity of meristematic cells, usually accompanied by an elevated endogenous auxin content in shoot apices may also inhibit virus replication. Thus, the meristem is highly protected from infection (Limasset *et al*., 1949). Based on this finding, meristem culture has been extensively used to eliminate viruses, bacteria and fungi from plants. The culture of meristems or alternatively small shoot tips, in combination with enhanced cell division *in vitro* and/or thermal pre-treatment allows the elimination of viruses in plants propagated from vegetative parts. If explants are too big, they are likely to contain virus particles in the associated vascular tissue.

For thermotherapy, the plants are first are grown at high temperature (ca. 38-42^0 C) for 4-6 weeks. Under tropical or subtropical conditions, this can be accomplished simply by installing a small compartment of a glasshouse equipped with a roof vent on one end and an exhaust fan on the opposite end, both temperature-regulated. This approach removes the excess heat and provides a constant high temperature daytime treatment. In climates with temperate conditions, the same effect can be achieved by placing fluorescent lights including ballasts and/or heat producing incandescent lamps in the necessary minimum distance to avoid damage over the plants to be treated in a dark box just large enough to include the plants. Such a system has been used for virus elimination in sweet potato (Schmidt and Grahsl, *unpublished*).

After thermotherapy, 0.2 –0.4 mm explants are preferentially cultured singly in test tubes. If the explant is too big, it likely has a vascular system that may contain microbial contaminants including viruses. The plants thus obtained are multiplied, and re-indexed. A better strategy is to culture 2-5mm long explants for 4-5 weeks, maintain the *in vitro* grown plant at high temperature for 4-5 weeks, and excise 0.2-0.4mm or even longer explants to initiate subcultures. This procedure avoids in vivo contamination problems and gives high

survival and multiplication rate. In potato, *in vitro* cultures were established from several millimeter long shoot tips and axillary buds after *in vitro* thermotherapy to free potato from all relevant virus diseases (Schmidt and Grahsl, *unpublished*). By using this method, it was possible to eradicate viruses A, Y and X from potato varieties in one single step (Ahloowalia, unpublished). A method called multiple lateral shoot technique of *in vitro* elimination of three common viruses, X, Y and S of potato, has been also reported (Zapata *et al*., 1995). In this method, a stem with at least five nodes is treated with ribavirin in liquid medium, and cultured for 5 days at room temperature. The stems are subjected to thermotherapy at 32-35^0 C for 25 days, apical buds are taken from the lateral shoots cultured on solid medium, and checked for virus infection with ELISA. The survival of explants in relation to the applied temperature stress and their size is always inversely proportional to the pathogen elimination success.

It is known from molecular and traditional microbiological surveys that effectively all *in vitro* plants contain certain microorganisms, mostly bacteria but also mycoplasmas, viroids, and fungi, many of which cannot be cultured without the host. Such microorganisms may not show symptoms, and in some cases may even have a positive effect on the growth performance of the host plant (Bell *et al*., 1995, Bensalim *et al*., 1998, Boddey *et al*., 1995, Cassells, 1997).

Shoot-tip culture is used for the multiplication of plants that are already freed from known diseases. It should be emphasized that it does not free the plant from viruses. In fact, it favors propagation of viruses and increases virus concentration in the daughter plants. In many ornamentals, variegation is due to the presence of certain viruses or mycoplasms (Lee et al., 1997). The removal of such viruses is therefore not desirable. In such ornamental plants, multiplication from shoot-tip and axillary bud culture is the better choice, because meristem culture may remove the virus, and the ornamental variegation is lost (Cassells *et al*., 1980). In certain varieties with variegations as in *Petunia* and *Pelargonium* meristem culture may dissolve the existing chimera, thus producing material without the desired character. Viruses may also produce certain phenotypes that are characteristic of a plant variety. For example, in sugarcane cv. 'Co 740', the yellow irregular leaf streaks were initially considered as a diagnostic character till they were proven to be disease symptoms. The removal of the virus by meristem-tip culture led to the disappearance of the yellow streaks, which reappeared on re-infection (Hendre *et al*., 1975). Erroneously, the yellow streaks are still recognized as a varietal diagnostic trait of 'Co 740'. Recently, endogenous para-retroviruses have been reported that integrate in the genome of the plant material (Harper et al. 1999). Under *in vitro* stress, sequences of such viruses have been detected in the micropropagated banana plants.

Perspective of disease freeness

Despite the fact that many viruses may not show visible symptoms, their presence can reduce the yield and quality of crops. Yield increases of up to 300% were reported following replacement of virus-infected stock with specific pathogen-free plants (Murashige, 1980). In China, yield losses from virus diseases of sweet potato (Feathery Mottle Virus, SPFMV, Latent Virus, SPLV and Caulimo-Like Virus, SPCLV) used to be as much as 20%. After the establishment of a virus elimination program, within a few years almost 10% area of 6.6 million ha under sweet potato is now planted with virus-free planting material (Gao *et al*., 2000).

There are no chemicals to cure virus-infected plants in the field. However, some less virulent viruses can protect plants from infection of their more virulent strains and create cross protection (Jones and Mullin, 1974). The phenomenon of cross protection need not

prevent the use of meristem culture. Under field conditions a crop does not stay absolutely virus free even though derived from tissue culture material. Even under tissue culture, total freeness may not be achieved.

Many symbiotic organisms contribute to the vigor and ability of the tissue-cultured plants to perform well in the field (Cf. Chapter 9). In the course of tissue culture, many plants are made free from such beneficial microbes, e.g. symbiotic nitrogen fixing endophytic bacteria, and mycorrhizae. There is no practical way to retain the beneficial microorganisms during tissue culture. The deliberate re-infection of propagules with selected strains can be a valid way of retrieving the benefits of such microbes (Cf. Chapter 9).

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QUALITY ASSURANCE

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Abstract. Quality checks are essential to assure production of high quality plants and to have end-users confidence. Quality standards require the establishment of suitable tests to maintain quality control. The choice of explant source, freedom of the donor plant from viruses, disease causing fungi, bacteria, viroids, phytoplasmas, vigour and conformity of the variety, and elimination of somaclonal variants are critical for maintaining plant quality. Variety identification by proper labeling at all stages is essential to ensure varietal identity.

INTRODUCTION

In many developing countries, there is a major need to provide large quantities of improved planting material to growers and farmers. Since quality checks in many developing countries do not exist beyond the laboratory gate, the assurance of high quality micropropagated plants is critical to have end-users confidence. Quality assurance becomes even more important when the plant material is for export. High quality of the super-elite and elite micro- and mini-tubers of potato has improved the production of quality seed potatoes in Ireland and Scotland where conventional multiplication had been inadequate to generate high quality disease-free planting material. Cost reduction based on low cost options is necessary for commercial micropropagation. While cost reduction is necessary for commercial micropropagation, the low cost option should not compromise quality. The sturdiness of plants is an important attribute of quality, which determines their ability to adapt to field conditions. The cost-effective systems should produce semi-hardened plants in the culture itself. Such plants produce a profuse and viable root system and adapt in less time to field conditions.

Quality standards require the establishment of suitable tests to maintain quality control and the assurance of micropropagated material irrespective of the system used. No industry can prosper without a quality policy based on carefully identified, relevant and recognized quality specifications and standard methods to test and verify them. Hence, the plants coming out of any facility should be subjected to quality testing without discrimination or prejudice. The commercial micropropagation industry, which is still in its infancy and early stages of evolution in many developing countries, lacks this vitally important component. Under the seed law, tissue culture plants are defined as “seed”. The specifications stipulated for seeds of cultivated crop plants do not apply to tissue culture plants for quality, except for the basic requirement of distinctiveness of the variety, genetic uniformity, and stability. This lack of fundamental specification creates conflicting opinions on the quality of micropropagated plants. Such a situation is undesirable for users, producers and regulating agencies and a hindrance to the maturity and growth of commercial micropropagation as a vibrant industry.

Several commercial micropropagation laboratories already follow standard practices such as labeling of cultures, use of indexed stock mother plants, and preventing secondary infection from pathogens during the hardening phase. The quality considerations need to take into account several parameters as a package. A comprehensive approach is needed, which will help micropropagators to monitor production quality, and formulate a quality- assurance system based on such tests and specifications, that are open to third party inspection. Such an

approach will also be useful to formulate well-defined quality specifications by regulatory authorities.

Factors affecting quality

The explant source (e.g. meristem tip, axillary buds), freedom of the donor plant from viruses, disease causing fungi, bacteria, viroids, phytoplasmas, vigour and conformity of the variety, and elimination of somaclonal variants are critical for maintaining plant quality. Many cultures are initiated from meristem-tip explants. Such cultures can be multiplied by enhancing axillary branching, which preserve genetic stability even on indefinite subculturing (Bhojwani and Razdan, 1983; Pierik, 1989). Since this method reduces the chance of differentiation of plants from cells other than the meristematic tissue, the expected genetic variation is low. However, the shoot-tip and bigger explant cultures have differentiated cells, in addition to the undifferentiated meristematic cells, and may result in somaclonal variants. Thus, shoot tip culture has a significant probability of producing genetic variants. However, in banana, the frequency of such variants markedly differs with the variety. Shoot-tip explants infected with endophytic microflora and viruses transmit them to daughter plants. Endophytic microflora is not desirable, unless it has symbiotic functions. Hence, meristem culture is the preferred method to initiate cultures for producing high quality plants.

Multiplication should be based on enhanced axillary bud proliferation, i.e. the rapid formation of tiny microscopic axillary buds in the axils of the leaves of the tiny plants that sprout and develop into daughter plants. The introduction of callus phase usually stimulated with growth regulators such as 2,4-D will introduce unwanted somaclonal variation and should be avoided during multiplication. For the same reason, the use of relatively high concentrations of growth regulators such as BAP, IAA, & BA should be avoided. Quality assurance begins with culture initiation, explant source, and judicious use of growth regulators during multiplication. Cultures initiated from meristem tip and multiplied through enhanced axillary branching avoid callus formation and give uniform clones. On the other hand shoot-tip cultures are considered inferior to meristem-tip culture since they can be the source of virus infection. In case of photoperiod-sensitive plants, light duration can also have a serious effect on the final performance of the micropropagated plants, particularly those meant for flower production. Hence, a neutral photoperiod of 12/12hr light/dark is preferred during multiplication.

Freedom from diseases

It is necessary to initiate cultures from the meristem-tip and ensure further multiplication free from viruses. For example, the badna virus in banana integrates into the host genome during tissue culture and can become a source of infection in micropropagated plants although parents are free from symptoms (Harper *et al*., 1999; Ndowora, 1999). If the parent plant is virus infected, the cultures from its shoot-tip will likely be infected. Even the meristem tip will carry over viruses unless thermo-therapy has been carried out and the cultures have been tested virus free. Removal of viruses will be positively undesirable in exceptional cases of ornamentals, where the characteristics are associated with the presence of viruses, as in the case of the yellow vein geranium.

The quality tests should be rapid, and aim at the identification of a variety or clones, and ignore point and bud-mutations, which occur in the normal population as a natural phenomenon. A variety is an assemblage of closely similar genotypes and clones, and not a group of “absolutely uniform” genotypes. Thus, the methods should be able to make a

distinction between two varieties, and not produce confusion with regard to intra-varietal variability.

The tests for quality include endophytic bacterial count. Broad range identification methods for the presence and quantification of endophytic bacteria are required and should be the first tests in quality control. This will prevent production of plants contaminated with undesired endophytic bacteria (Cf. Chapter 9), and may indirectly be a useful indicator of the absence or of low concentration of viruses. Conventional bacteriological methods are enough to standardize such a test. PCR based methods may provide identification of a wide range of bacteria. For the identification of viruses, light microscopy for inclusion bodies has limited sensitivity. Immunology based methods have been commonly used for detecting specific viruses e.g. in potato. However, PCR methods are being increasingly developed and finding increasing use.

Labeling and culture identity

Micropropagated varieties in culture and at the hardening stage are difficult to distinguish visually from one another. Variety identification by proper labeling at all stages is essential to ensure varietal identity. When several varieties of the same plant species are used, accidental mislabeling or mixing may occur. Therefore, monitoring is essential to detect admixtures at all stages. Clones of the mother variety should be maintained by conventional vegetative propagation to fall back upon whenever necessary.

Varietal conformity

Varietal conformity based on morphological characters has been the basis for identification in conventional propagation and the same applies to the plants derived from tissue culture. However, considerable caution is advised in selecting morphological characters for varietal conformity. For example, in sugarcane, variation in morphological characters such as cane colour, shape of the bud, size of the ligule can vary in the different parts of the same cane.

Where morphological characters of mother plants of diagnostic importance are due to interaction with microbes, meristem tip culture invariably gives rise to a new phenotype due to removal of such microbes. In such cases, the new virus free phenotype represents the original expression of the new virus free phenotype (Cassells *et al*., 1980, Hendre *et al*., 1975). Hence, criteria other than morphology are necessary for varietal conformity. Morphological characters are not an absolute proof of genetic conformity or genetic stability in tissue culture. Many times, physiological changes may lead to the appearance of types among micropropagated plants that differ from the parental clone. In some cases, such variants with minor phenotypic changes can be used for field production, but not for propagation. Low frequency mutations and bud mutations occur in conventional populations propagated from vegetative parts. Genetic changes not associated with morphological traits can be detected by DNA based molecular methods. Somaclonal variants are undesirable in cloning, but can be used for breeding.

DNA based molecular markers

DNA based molecular markers are not affected by the environment and are more reliable than morphological characters for identification of varieties (Weising *et al*., 1995) but are not a low cost option for small facilities. Hybridization and RAPD (Randomly Amplified

Polymorphic DNA) based methods can discriminate between varieties and clones. However, the differences between the donor plant and those regenerated through tissue culture cannot be detected with such methods. Only in a few cases, it is possible to discriminate the mother plants from the variant regenerated plants (Poulson *et al.,* 1993; Nelke *et al.,* 1993). Molecular methods appear to be reliable for discrimination at the variety level.

Variety identification based on DNA fingerprinting, such as RAPD, microsatellites, STR's (Sequence Tagged Repeats), AFLP (Amplified Fragment Length Polymorphism) and multi-locus probes, is potentially useful. RAPD can be standardized within a short time without elaborate knowledge of DNA sequence. However, limitations in the reproducibility of this method (between two sets of experiment, two machines, two workers and two laboratories) are well recognized. This limitation can be overcome by including a sample of reference DNA. Epidermal patterns and structures and stomata, if stable in tissue culture, are additional tools in variety diagnosis. If their validity is confirmed, they may be simpler than RAPD and other molecular methods.

Cost and quality

For small facilities, molecular tests are expensive. For them, it is cheaper to obtain the services of large or centralized laboratories (e.g. departments of agriculture and universities), which can either index cultures for a fee or supply indexed mother cultures at regular intervals. Unequivocal tests can help to develop in-house quality control systems. Such tests can be used for developing third party inspection systems, and provide a practical basis for statutory quality control systems.

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PRIMING TISSUE CULTURED PROPAGULES

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Abstract. Priming of *in vitro* propagules refers to the manipulation of the growing environment, prior to and upon transplanting, and is an integral part of tissue culture propagation. Tissue-cultured propagules are produced under controlled environment. Such plantlets have small juvenile leaves with reduced photosynthetic capacity, and malfunctioning stomata. Priming for rooting, shooting, and improved photosynthesis can be achieved with growth regulators and simple adjustment to the growing conditions that affect the post-transplanting performance of the propagules. Vented closures with microbial filters have been used to facilitate gas exchange to reduce ethylene build-up that stunts plant growth, reduces leaf-size, and causes leaf drop in tissue culture containers. Plantlets produced under photoautotrophic culture systems on media with or without sucrose but CO_2-enrichment, increased light intensity, good gas exchange and reduced humidity are more vigorous, have larger root- systems, and are less susceptible to microbial contamination. Plants adapted gradually to the *ex-vitro* environment have improved survival upon transfer to soil. Plants in their natural environment are colonized with many bacteria, fungi, and mycorrhizae. *In-vitro* or *ex-vitro* biopriming of micropropagated plants with such organisms can improve plant performance under stress environments, and consequently enhances yield.

INTRODUCTION

The adaptation of plants to the environment - temperature, osmotic stress, and pathogens, is triggered by modification of expression of specific genes and metabolic pathways. Certain chemicals, environmental factors, and microorganisms pre-sensitise the cellular metabolism of plants. Upon exposure to stress, the pre-sensitised or primed plants adapt better and faster than the non-primed plants (Conrath *et al*., 2002). The production of high quality and vigorous plants through *in vitro* culture requires the enhancement of post-transplanting ability for water management, efficiency of photosynthesis and resistance to diseases. Priming of *in vitro* propagules, based on manipulation of the growing environment (chemical, physical and biological) prior to and upon transplanting, is an integral part of tissue culture propagation. The ability of the propagules to withstand transplanting stress very often determines the success or failure of tissue culture operations.

Tissue-cultured propagules are produced in controlled environments under pre-set temperature and low-light intensity, and in small vessels with poor air exchange. This leads to high humidity and accumulation of ethylene (Jeong *et al*., 1995). Also, the nutrients in the growing media, sucrose, mineral salts, vitamins and growth regulators, are far in excess of those in the soil. Such an environment causes developmental distortions, and represses or modulates several metabolic pathways. As a result, the plantlets have small juvenile leaves with reduced photosynthetic capacity, and malfunctioning stomata. The roots of such plants lack hair or have few, and the leaves have poor cuticle development and low wax deposit (Pierik, 1987; Ziv, 1991).

Priming for rooting

The ability to manage water, photosynthesis and response to stress during and after *in vitro* culture determines the final performance of the propagules. The formation of a functional root system is an essential step to manage water under the *post vitro* conditions. The *in vitro* roots remain functional and continue to grow during *ex-vitro* acclimatisation. Easy to root species, (e.g. potato) usually regenerate roots upon subculture on hormone-free medium. However, root architecture and biomass partitioning between roots and shoots can be easily manipulated by simple adjustment to the growing conditions that affect the post-transplanting performance of the propagules. For example, increasing day/night temperature from 20/15 to $33/25^0$C either slightly stimulates or has no effect on the shoot growth in clones of potato, but dramatically enhances root growth and root branching (Bensalim *et al*., 1998).

Several chemicals also influence the development of the root system. Abscisic acid is known to affect initiation of new lateral roots and overall changes to the root architecture (Hooker and Thorpe, 1998). Jasmonic acid (JA) has a similar effect. Low JA concentrations (0.5-1.0µM) stimulate both root and shoot growth (Fig.1). Jasmonates induce stress resistance in plants (Conrath *et al*., 2002), and may be useful for priming of *in vitro* plants during the last subculture. In potato, nodal explants from JA primed stock plants tuberize earlier and more uniformly, and produce more microtubers than the non-primed controls (Pruski *et al*., 2002). Salicylic acid (SA) is a major signalling compound and induces abiotic and biotic stress resistance in plants (Conrath *et al*., 2002). It also induces adventitious roots in cuttings and stomata closure, and inhibits ethylene biosynthesis in detached leaves (Huang *et al*., 1993); hence it may also be useful for priming tissue-cultured propagules.

Many woody and herbaceous perennials require auxins for rooting. Auxins are involved in root initiation and adventitious root formation (Klerk *et al*., 1997). However, excessive auxins after root induction may inhibit root regeneration and elongation, and impair formation of a good root system. Therefore, a careful selection of the auxin type and concentration is recommended. IAA and IBA are usually used for easier-to-root plants and NAA for more recalcitrant woody plants. The efficacy of different auxins also depends on the explant type, and exposure to light. For example, a short duration (3-7 days) of dark incubation enhanced rooting in apple (Klerk *et al*., 1997), and papaya (Yu, *et al*., 2000).

Addition of low concentration of the growth retardant daminozide to hormone-free medium enhanced post-transplanting acclimatisation of potato plants (Tadesse *et al*., 2000). Other supplements like activated charcoal, seaweed concentrate, pyroligneus acid, and dilution of salts in the basic MS medium can also aid root initiation and growth (Pierik, 1987). *In vitro* cultured papaya shoots in aerated vermiculite gave better rooting with higher post-transplanting survival than on non-aerated vermiculite and non-aerated agar (Yu *et al*., 2000).

In some difficult-to-root species, e.g. apple and Saskatoon berries (*Amelanchier alnifolia* Nutt.), *in vitro* rejuvenation via several subcultures is required before successful rooting of microcuttings. Individual cultivars may require adjustment to culture conditions and different number of subcultures. For example, over 90% rooting was achieved with apple microshoots, cv. 'Jonathan' by the ninth subculture, but a more recalcitrant variety 'Red Delicious' required over 30 subcultures for successful rooting (Howard and Marks, 1987). In Saskatoon berries, where rooting by traditional stem cuttings is very difficult, tissue culture rejuvenation and subsequent transplanting to the greenhouse can generate highly vigorous plants. Shoot cuttings taken from such planted hedges readily root within 2-3 weeks after a simple treatment with IBA (Pruski *et al*., 1990).

0.5 1.0 1.5 5.0 10 25

Jasmonic acid concentration (μM)

Figure 1. Five-week-old potato plants cv. 'Kennebec' grown on 10 ml hormone-free MS medium (pH 5.7, 3% sucrose, vitamins and 0.6% agar), supplemented with various concentrations of JA at 22/18ºC day/night temperature, 16 h photoperiod, 150 $\mu E^{-2}s^{-1}$ fluorescent light.

Increasing photosynthetic competence

Tissue culture containers provide sterile conditions and high moisture, thereby preventing contamination and desiccation of the explant, but restrict aeration and escape of metabolically generated ethylene and water vapors. Such conditions slow down growth and induce undesirable morphological changes. The ethylene build-up stunts plant growth and reduces leaf-size, and causes leaf drop. Such plants desiccate quickly upon transfer to the external environment. Vented closures, including those for baby-food-jars with built-in different sizes of microbial filters, have been used to facilitate gas exchange between culture vessels and the ambient environment.

High relative humidity in culture vessel reduces deposition of epicuticular waxes on leaves. It impairs the functioning of stomata and alters leaf mesophyll by reducing the number of palisade cells and by forming spongy parenchyma with large air spaces. Other effects include low nutrient uptake due to decreased transpiration, decreased lignification, and reduced or abnormal development of the vascular system (Debergh *et al.*, 1992; Kozai, 1991; Ziv, 1991). Although the cuticular wax development and stomata functioning can be

induced by gradual adjustment to lower humidity, morphological changes cannot be easily reversed. The altered leaf morphology reduces photosynthesis and enhances respiration. Cooling the bottom of culture vessels by 2-3^{o}C below the ambient air temperature in the culture room or providing high light intensity can reduce high humidity. Gradual opening of vessels for a few days prior to transplanting can also adjust high humidity (Ziv, 1991).

Many micropropagated plants have C_3 photosynthetic pathway; hence, are greatly affected in their ability to assimilate carbon. The balance between photosynthesis and photorespiration controls their productivity (Ku *et al.*, 1977). Under the traditional methods of micropropagation, cultures are usually mixotrophic (use carbon source mostly from sucrose in the medium as well as photosynthesis). The carbohydrates in the media inhibit carbon assimilation via photosynthesis by reducing rubisco (ribulose-1-5-bisphosphate carboxylase/oxygenase) activity (Kozai *et al*., 1995). On the other hand, several studies indicate a stimulatory effect of sugar on biomass accumulation under elevated CO_2 (Nowak *et al*., 1999).

In the photoautotrophic culture systems the cultures are grown under CO_2-enrichment (1000-1500µmol mol^{-1}), increased light intensity (at least 150µmol m^{-2}, s^{-1}), good gas exchange and reduced humidity, and on media with or without or sucrose (Kozai *et al*., 1997). Plantlets produced under such conditions are more vigorous, have larger root- systems, and are less susceptible to microbial contamination (Jeong *et al.,* 1995). They are able to photosynthesize, and do not require acclimatization during post-*vitro* establishment.

Priming for temperature tolerance

In vitro grown plantlets can be acclimatized to low temperatures for cold tolerance under field conditions. Alfalfa and red clover seedlings withstood -16 and -10^{o} C, respectively, upon cold hardening at 2-5^{o} C, 80µE m^{-2}, s^{-1} light and 8h photoperiod, for 3-5 weeks. Such hardening has been successfully utilized in the selection of cold tolerant alfalfa and red clover genotypes (Nowak *et al*., 1992). Elevation of sucrose in the culture medium or application of abscisic acid, or both, may further improve plantlet hardiness in some species (Chang and Reed, 2001).

Acclimatization to soil transfer

Good quality propagules with well developed roots and leaves are easy to acclimatize to the external environment. Any successful acclimatization protocol must ensure that the plants maintain active growth during the entire weaning period (McCown, 1986). The effect of *in vitro* growth conditions on post-*vitro* acclimatization has been demonstrated in grapevine (Amâncio *et al*., 1999) and chile ancho pepper, *Capsicum annuum* L. (Estrada-Luna *et al*., 2000). The most common approach to improve plant survival upon transfer to soil is their gradual adaptation to the *ex-vitro* environment. Under such conditions, plants rapidly convert from heterotrophic or photomixotrophic to autotrophic growth, form fully functional root systems, and their stomatal and cuticular transpiration is reduced (Ziv, 1986). Such gradual adaptation is often carried out in greenhouses by decreasing relative humidity using fog or mist chambers and increasing light intensity and shading techniques. Light intensity needs to be adjusted to the plant requirements. Rooting of *in vitro* produced shoots *in vivo* is recommended for some species to promote development of roots already adapted to external conditions, to prevent potential damage to fragile *in vitro* roots, or to avoid post-rooting dormancy. Other treatments of *in vitro* plantlets upon transplanting to the outside environment include high phosphate fertilizer to enhance their vigor, fungicides to prevent damping-off

(Debergh and Read, 1991) and anti-transpirants to prevent desiccation; however, they may cause phytotoxicity (Jeong *et al.,* 1995).

A simple, low cost method for *ex vitro* weaning and liner production of woody plant species and Hosta has been developed (Pruski, K.*, unpublished*). The shoots with root-initials from the rooting medium are transplanted in 'Jiffy'^{TM} plugs (Fig. 2). Plants are acclimatized in plastic trays (25x50x5cm) with transparent cover. Each tray holds 300 plugs, each 18mm diameter. The plugs are saturated in a 2.5-3L solution of fertilizer (1g/L) 10-52-10 NPK; the excess solution is discarded before transplanting. The plants are watered weekly (approx. 1L water/week) and kept in the growth room benches for 3-weeks after transplanting at 22-24/20ºC day/night, 14h photoperiod, under 120 $\mu Em^{-2}s^{-1}$ fluorescent light. The current cost is US$73 for 5400 plugs, $58 for 100 trays, and $60 for 100 plastic covers; the total cost per plug being US$0.018. The rooting success in woody plants, e.g. Swedish columnar aspen, Saskatoon berry, chokecherry and pincherry cultivars, sour cherry, low bush blueberries and others was more than 95%, and 100% with hosta varieties.

In seed potato production, 2 to 4-week old tissue cultured plantlets are usually hardened off for 2-4 weeks in a greenhouse, and transplanted in the field. Such plantlets are still juvenile and not well adapted to stress. To prevent desiccation, insect and bird damage, and spread of viruses by aphid, many growers cover plants with floating covers and install an overhead irrigation system. Seed potatoes produced under floating covers are consistently better in quality than those from the unprotected planting.

Figure 2. Plant acclimatization in plastic trays with transparent cover. Left. Trays in a growth room bench next to culture vessels. Right. Aspen (Populus tremula var. erecta) liners in Jiffy plugs, three weeks after transplanting.

In vitro produced-propagules of woody species, e.g. Saskatoon berry (*Amelanchier alnifolia* Nutt.), Chokecherry (*Prunus virginiana* L.), Pincherry (*P. pennsylvanica* L.), Swedish columnar aspen (*Populus tremula var. erecta*), European weeping birch (*Betula pendula var.* '*Gracilis*' become dormant after rooting. *In vitro* rooted Saskatoon berry is particularly prone to dormancy as stress factors readily induce terminal bud dormancy. When the shoots stop growth, leaf shedding occurs in the culture or soon after transplanting, and the propagules desiccate. The liners produced from such plants are less vigorous and difficult to establish in the field. In Chokecherry (*P. virginiana* L.), dormancy symptoms of shoots with

reddish leaves and cessation of terminal-bud growth are induced upon depletion of nutrients in the medium. Post-rooting dormancy has also been reported in rhododendrons and low bush blueberries. Adding low amounts of gibberellic acid (GA) to the medium prevents dormancy, although shoots treated with GA are difficult to root (Pruski *et al*., 2000). In Saskatoon berry, e*x-vitro* rooting under non-sterile conditions is helpful, although does not eliminate the problem. Spraying leaves of *ex vitro* rooted-shoots with BAP (400ppm), followed by GA_{4+7} (100-PPM) the next day prevents dormancy (Pruski *et al*., 1990).

Priming somatic embryo-derived plants

Somatic embryos are produced as adventitious structures directly on explants of zygotic embryos, from callus and suspension cultures (Cf. Chapter 1). The stress inducing molecules in plants, ABA and JA have been successfully used to facilitate embryo maturation and priming for resistance to post transplanting stress. ABA and osmotic shock with 6% sucrose is a routine step to induce desiccation tolerance in alfalfa synthetic seeds. Osmotic stress induction with 3% sorbitol was effective in embryo maturation and conversion in soybean cultures (Walker and Parrott, 2001) and ABA treatment combined with partial desiccation of encapsulated somatic embryos in sugarcane (Nieves *et al*., 2001). Several sugar alcohols and polyethylene glycol have been tested with different degrees of success for priming SE propagules of various plants (Walker and Parrott, 2001). In conifers, post ABA embryo maturation has been significantly improved by benzyl adenine (BA) (Schuller *et al*., 2000). The lowering of sucrose concentration from 58 to 29mM in the proliferation medium improved the formation of cotyledonary SE on the maturation medium. Interaction between plant growth regulators and various carbohydrates and organic nitrogen compounds in embryo development and maturation has been reported (Schuller *et al*., 2000).

BIOPRIMING

Plants in their natural environment are colonized both by external and internal microorganisms. Some microorganisms, particularly beneficial bacteria and fungi, can improve plant performance under stress environments, and consequently enhance yield (Brown, 1974; Lazarovits and Nowak, 1997; Creus *et al*., 1998). Plants infected by microorganisms develop systemic resistance (systemic acquired resistance, SAR, or induced-systemic resistance, ISR), and/or benefit from their antagonistic abilities towards pathogens (cross protection). Although, the inoculation of seeds with beneficial microorganisms has been practiced for more than 50 years, the inoculation of tissue culture propagules to enhance plant performance is relatively new. Plant tissue culture is based on axenic (contaminant-free) culture systems. Hence, microorganisms, including endophytes, are treated as problem-causing contaminants, and various procedures have been developed to eliminate them. Only recently, have microbial inoculants, primarily bacterial and mycorrhizal, been evaluated as propagule priming agents both as *in vitro* co-cultures and on transplanting.

Standard microbiological techniques allow culturing only a few percent of the naturally occurring microorganisms. Moreover, the soil and plant associated bacteria can switch between culturable and unculturable stages. The development of new culture methods that allow the establishment of stable associations between plants and beneficial organisms, both *in vitro* and *ex vitro*, under different environments is a major challenge of future research.

In vitro bacterization

Rhizosphere bacteria interact with plants and other inhabiting organisms by producing a wide array of chemicals. Many of these chemicals exhibit plant regulatory activity. There is increasing evidence that the bacteria associated with plants (endophytic and epiphytic) induce host resistance to environmental stresses. This is commonly linked to the production of salicilate or jasmonate, or both (Conrath *et al*., 2002). These two types of signalling chemicals are associated with resistance to biotic stress. Recent molecular evidence in *Arabidopsis thaliana* inoculated with the plant growth promoting rhizobacterium, *Paenibacillus polymyxa,* indicates however, that the abiotic and biotic stress-resistance are linked.

An effective plant beneficial bacterium, *Pseudomonas* spp. strain PsJN, was used to enhance tolerance to transplanting stress in potato. The bacterium was originally isolated as a contaminant from *Glomus vesiculiferum* infected onion roots. A key element in its isolation was that the bacterium (*Pseudomonas* sp. strain PsJN) did not grow on a standard potato culture medium in the absence of plantlets. Pre-selection of plant beneficial bacteria on plant tissue culture media is thus recommended as a critical step in the development of an *in vitro* co-culture system, so the bacteria do not overgrow the culture.

Dipping potato nodal cuttings in a suspension of a beneficial bacterium can dramatically affect *in vitro* growth of plantlets. So far, our isolate, *Pseudomonas* sp. strain PsJN, has been the most effective plant growth promoting bacterium under *in vitro* conditions (Conn *et al*., 1997; Nowak, 1998). It forms endophytic and epiphytic populations when co-cultured either with potato, tomato and grapevine. Thus, in clonal propagation by nodal explants, there is no need for further re-inoculation. The bacterium stimulates plant growth and induces changes that lead to better water management. It also enhances resistance to low level pathogens of potato and tomato (Lazarovits and Nowak, 1997; Sharma and Nowak, 1998) and to *Botrytis cinerea* infection of grapevine (Barka *et al*., 2002). The inoculated plantlets had a massive and well-branched root system after four weeks in culture, and were more advanced developmentally (Frommel *et al*., 1991). The stems were sturdier with more lignin deposits around the vascular system, more root hairs and more and larger leaf hairs (Nowak, 1998). Stomata function of the bacterized *in vitro* plantlets closely resembled those of the greenhouse hardened non-bacterized transplants. In wheat seedlings exposed to osmotic stress, improvement of water relations has been observed by their co-culture with *Azospirillum brasiliense* strain SP245 (Creus *et al*., 1997). Enhanced transplant survival of black locust (*Rabinia pseudoacacia* L.), inoculated with different *Rhizobium* isolates selected for growth vigour from *Rabinia* varieties, has also been reported (Balla *et al*., 1997).

The stimulation of adventitious rooting in cultures with *Agrobacterium rhizogenes* is well known. It was successfully used to enhance rooting of two Mexican species of *Pinus* (Villalobos-Amador *et al*., 2002). Rooting of somatic embryo-derived adventitious shoots (15-30 mm long) increased between 7-13%, and in shoots co-cultured with *A. rhizogenes* to 67% compared with 60% in the auxin treated controls. Root development was also induced in slash pine explants on co-culture with the bacterium isolates (Burns and Schwarz, 1996). The bacterium-induced roots resembled seedling roots rather than hairy roots, which are induced with *A. rhizogenes*. Induction of root elongation has been observed in potato plants co-cultured *in vitro* with some strains of *Rhizobium leguminosarum* biovar *trifolii*, isolated from the root nodules of red clover (Fig. 4).

The bacterization of shoot explants of oregano with a *Pseudomonas* sp. prevented vitrification. The oregano plantlets co-cultured with the bacterium had lower water content, but more phenolics and chlorophyll than the non-bacterized controls (Shetty *et al*., 1995).

Similar responses were recorded with potato and vegetable crops. Bacterized plantlets were greener, had elevated levels of cytokinins, PAL (phenylalanine ammonia-lyase), free phenolics (especially chlorogenic and caffeic acids) (Nowak *et al*., 1997), and contained more lignin. In potato, elevated PAL activity in bacterized plants was found during the first four weeks of growth, there being no difference in its level in six week-old plants. However, when explants were taken from six-week-old bacterized and control stock plants, PAL activity in the newly derived shoots was higher in the pre-bacterized treatment (Nowak *et al*., 1997). This suggests that pre-bacterized plantlets respond faster to cutting injuries, a critical factor in the induced host resistance to pathogen attack.

In vitro bacterization of potato and vegetable plantlets significantly enhanced their post transplanting survival vigour. Enhanced vigour of potato plantlets co-cultured with isolates of *Pseudomonas fluorescence* isolated from tubers, during and after weaning has also been reported (Duffy *et al*., 1999). In greenhouse experiments, plants derived from dual cultures of potato and our pseudomonad bacterium had larger root systems and stolons, tuberized earlier, and gave higher tuber yields than the non-bacterized controls (Dunbar, 1997). The bacterized potato plantlets transplanted directly from culture vessels to the field had significantly better survival than the non-bacterized controls (Nowak *et al*., 1999); however, the tuber yield varied from year to year; high precipitation or severe drought reduced the benefits of bacterization.

Tissue culture techniques provide an opportunity for the introduction of nitrogen fixing endophytes (other than *Rhizobia*) into clonally propagated plants for sustainable production systems. A protocol has been developed to inoculate micropropagated sugarcane plantlets with *Acetobacter diazotrophicus* at the end of the rooting period on medium with 10X diluted salts and sugar but without hormones and vitamins. Thirty days after transplanting, the bacteria could be re-isolated from the plant tissues (Reis *et al*., 1999). Stable artificial associations between plants and nitrogen fixing bacteria have been also reported. A symbiotic culture system of callus and bacteria between *Daucus carota* L. and *Azotobacter zettuovii* (CRS-H6) was developed. The callus grew for four years on the nitrogen-free medium with lactose as the carbon source. The bacteria located in the intracellular spaces were transmitted to newly regenerated plantlets and fixed nitrogen (Varga *et al*., 1994). Similar stable associations were established with tomato, potato, wheat, sugarcane and poplar, using different strains of eight *Azotobacter* species (Varga, S.S. *Personal communication*), and in strawberries with *Azomonas insignis* (Preininger *et al.,* 1997). In tomato, the bacterium was transmitted through the seed. Approximately, 20% of the seedlings from the seed of tomato cocultured with *Azotobacter beijerinckii* showed N_2 fixing activity two months after germination (Varga, S.S. *Personal communication*).

Ex vitro bacterization

To comply with the certification requirements of plant tissue culture propagules, commercial laboratories are hesitant to introduce microorganisms into their *in vitro* cultures. One approach is to wean plants in rhizosphere with both beneficial bacteria and mycorrhizal fungi. In potato, the tissue-cultured plantlets are readily infected both in external and internal tissues with bacteria present in the transplanting medium. This approach, although attractive, will have to deal with the problem of maintaining stable populations of the introduced microorganisms. The beneficial effects of bacterial inoculation on tree seedlings (Enebak *et al*., 1998) and greenhouse produced potato tubers (Nowak *et al*., 1999) show the potential of post-*vitro* bacterization of tissue-cultured propagules. The post-*vitro* mycorrhization and bacterization of micropropagated strawberry, potato and azalea with certain combinations of bacteria and mycorrhiza enhanced greenhouse production of minitubers, and a mixture of

three strains of rhizobacteria improved the post-transplanting performance of strawberries (Vosatka *et al*., 2000).

Mycorrhization

The inoculation of tissue-cultured plantlets with endomycorrhizal fungi is beneficial to plant growth. It is well known that the root colonization with vesicular-arbuscular mycorrhizae (VAM) improves plant nutritional status, water management and disease resistance (Fortin *et al*., 2002). Several secondary metabolites (cyclohexenone derivatives) with structural similarity to abscisic acid have been identified in tobacco roots in response to the *Glomus intraradices* infection (Maier *et al*., 1999). A variety of phenylpropanoid compounds induced by ectomycorrhizal inoculants, have also been identified in several conifers. Such compounds are related to the biotic and abiotic stress management in plants.

To improve the performance of tissue-cultured plantlets, rooted-shoots have been inoculated with mycorrhiza at transplanting. The benefits of mycorrhization depended on the growing medium, plant and mycorrhizal species, and the degree of root colonization. Because VAM stimulated growth of plants was frequently expressed only after acclimatization, it was suggested to develop a culture system where the mycorrhizal fungi are introduced *in vitro* during the rooting stage (Wang *et al*., 1993). It is possible to grow fungi *in vitro* on Ri T-DNA transformed carrot roots under elevated CO_2. Capitalizing on this technique, a 'tripartite' culture system for *in vitro* inoculation of strawberries (Desjardins, 1995) and vegetable crops was developed. The most effective treatment consisted of 30 day-old VAM (*Glomus intraradices*) transformed carrot-root culture and strawberry shoots in cellulose plugs. After root induction, the plugs were placed on the surface of the mycorrhized root culture and kept in a growth chamber under 5000 PPM CO_2 for 20 days. All plantlets were successfully colonized, and exhibited a larger root system, better shoot growth, and a higher (more negative) osmotic potential when compared to non-mycorrhized controls. It is suggested that the enhancement of osmotic potential is important in the pre-adaptation step prior to full acclimation of plantlets for transplanting. The high osmotic potential of wheat seedlings co-cultured with *Azospirillum* allowed them to withstand osmotic stress much better than non-inoculated controls with low osmotic potential (Creus *et al*., 1998). In strawberry, mycorrhized plants had a better establishment rate and produced more runners than non-mycorrhized controls (Murphy *et al*., 1997). The plants were grown in polyurethane foam substrate under photoautotrophic conditions with reduced sucrose concentration in the medium. The foam tear-away strips fit into any culture vessel, and the VAM spores can be placed directly in the planting holes. In garlic, improved growth was observed after *post-vitro* transplant inoculation (Lubraco *et al*., 2000). Increased rooting and reduction in weaning stress have been reported in the medicinal plant, *Babtista tinctoria* (L.) R. BR. (Grotkass *et al*., 2000).

Several studies have demonstrated synergistic effects of VAM fungi and diazotrophic bacteria on nutrition and growth of various crops (Paula *et al*., 1992). Such effects of "mycorrhiza helper bacteria" need to be explored in tissue culture systems. Creation of defined tissue culture microecosystems could allow the study of complexed plant–microbial-environment interactions, which could refine and improve our traditional *in vitro* propagation methods and prime the propagules for *ex-vitro* environments.

Priming with microbial derivatives

Cyanobacterial and fungal culture extracts have been shown to induce developmental changes and pathogen resistance in tissue-cultured material when added to the medium. Microbial culture filtrates have been reported for the control of plant tissue culture contaminants (Hussain *et al*., 1994). An acetone precipitated fraction of *Bacillus subtilis* Ehrnberg (strain 2) and *Trichoderma viride* Pers. (strain A) exhibited high antifungal activity, and extracts from *Pseudomonas fluorescence* Migula (strain X) had high antibacterial activity. The bacterial extracts did not reduce the performance of cultures of *Nicotiana tabacum* L. over four subcultures but reduced the growth of accidental contaminants. Heat-stable mycelial extracts of another *Trichoderma* species, a non-pathogenic *T. longibrachiatum* were triggered induced-resistance response to *Phytophthora parasitica* var. *nicotianae* (race 0) in tobacco seedlings (Chang *et al*., 1997). The extract induced expression of pathogenesis-related proteins, PR-16 and osmotin (PR-5) at a higher level than the extract prepared in the same manner from the pathogenic fungus.

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INCORPORATION OF LOW COST OPTIONS

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Abstract. For many plants, the conventional methods of propagation are cheaper than tissue- culture technology. Low cost options can generally be incorporated into the design of the building, laboratories, working areas, layout of equipment, lighting, heating and production planning to provide smooth and efficient operations. It is important to select several plants that provide options for production around the year to allow cash flow and optimal use of equipment and facilities. It is essential to maintain sufficient mother cultures, and limit the number of subcultures to avoid variation, and plan production and shipment according to the customer's demand.

INTRODUCTION

Low cost options can generally be incorporated into the design of the building, laboratories, working areas, layout of equipment, lighting, and heating to provide smooth and efficient operations. However, some options are stage-specific to the process of micropropagation and others are linked to marketing. The following strategies should be considered before venturing into commercial micropropagation.

Selection of crops

Micropropagation protocols have been developed for a large number of species; however, only a limited number of plants are being produced on a large scale through micropropagation. This is because conventional methods of propagation are cheaper than tissue culture technology or there is a selective market demand. Ornamentals account for approximately 80% of the world trade in the tissue culture industry. Plants produced by commercial laboratories should yield steady revenue, e.g. *Spathiphyllum*, *Syngonium*, lilies. Some plants are unique to individual commercial companies. The life cycle of the species and the cropping system adopted by growers are important criteria to select plants for large-scale tissue culture. Initially, micropropagation units focused on the production of ornamental plants that were least affected by the season, e.g. foliage and indoor plants. But to capture larger markets, commercial tissue culture units have ventured into fruits, vegetables, landscape and medicinal plants, which provide options for production around the year. This leads to improved cash flow and optimal use of equipment and facilities. When selecting specific varieties protected under Plant Breeders Rights, these should be multiplied only with the agreement of the breeders.

Target markets

Before going commercial, one must ensure that there is a large and expanding market for the product. Both the domestic and export markets should be explored extensively. It must be ensured that large contracts are available each year. Products that command a high price should be preferably chosen.

Regularity of despatches

It is essential to plan the production schedule according to the customer's demand and despatch the products at the stipulated time. It is not in the interest of the tissue culture facility to remain idle. Production of different plants should be distributed over the year to make optimum use of the laboratory facilities. Regularity of despatches adds to the viability of the business.

End product

It is essential to decide on the type of products to be produced in a tissue culture facility. These could be plants that are rooted or non-rooted and could be sold in agar or marketed as hardened propagules in plugs or trays. Relatively small tissue culture units can produce non-rooted microcuttings in large numbers. Rooted microcuttings on the other hand require large growth room areas. In both cases, dispatching them ex-agar may result in some mortality at the grower's end. To reduce mortality, it is important to ship them in-agar. However, in-agar shipments occupy a large volume, and hence require more cargo space and add to the cost. Such high transport and cargo charges can only be justified for high value products. The hardened micropropagated plants in plugs or trays are the easiest to handle by growers.

Prevention of variation in tissue culture

The establishment of mother cultures from explants is a significant part of the production cost. Production projections can get out of gear due to the lack of sufficient mother cultures and maintenance of large numbers of such cultures adds to the cost of production. There is a general consensus that the number of subcultures for explants should be limited to avoid somaclonal variation. For example, in potato, the number of subcultures is recommended to be around twenty (Ahloowalia, 2000). Hence, repeated explant culture initiation becomes necessary to have sufficient number of mother cultures whenever large-scale production is planned.

For many plants, it is not known whether limiting the number of subcultures reduces genetic variation. Many of the plants that are propagated from vegetative parts such as banana, potato, sweet potato, sugarcane, chrysanthemum and many fruits are polyploids and well buffered against the expression of mutations and somaclonal variation. If axillary bud proliferation is used as the strategy of multiplication, the frequency of variation is low. If variation does occur, the number of variants will rise with increasing number of subcultures unless there is selective elimination in tissue culture. The high frequency of somaclonal variation has prevented the use of tissue culture for mass scale propagation of oil palm. In general, the initiation of cultures from meristem-tip gives low variation in subcultures. Some of the novel somaclonal variants can be of value in expanding the market (Jain *et al*., 1998).

Multiplication and hardening

Liquid media reduce the costs of multiplication. Increasing the multiplication rate through enhanced axillary bud proliferation helps in cutting cost of production. Mechanical cutting and bioreactors is a valuable option at Stage II (Cf. Chapter 6). In some systems, shoot elongation and rooting are two separate phases. Combining them in one step by using appropriate media with auxins and cytokinins is a valid cost cutting strategy. Reducing the duration in the hardening facility but increasing the survival rate reduces production costs. If natural light is used during multiplication and rooting, the plants need relatively shorter

duration for hardening. Acclimatization *in vitro* helps in better survival when the plants are transferred to soil (Ziv, 1986). Loss of plants at the hardening stage contributes to increased costs of production. A cost-effective system based on natural sunlight for *in vitro* acclimatization gives high survival (Savangikar *et al*., 2002). If grown under artificial lighting, tissue cultured plants should be kept under shade for some time and only then subjected to full sun hardening.

Greenhouse facility

Sophisticated and costly greenhouses can be substituted by cost-effective techniques for hardening *in vitro* derived plantlets. For this, it is necessary to understand the changes that tissue-cultured plantlets go through during the transfer from *in vitro* to *in vivo* conditions (Table 1).

Table 1**.** Changes in the environment for hardening *in vitro* plants to *ex-vitro* conditions

In vitro conditions	*Ex-vitro* conditions
Temperature 25 ± 2° C	Temperature 23-36° C
Light intensity 1200-2000 lux	Light intensity 4000-12000 lux
Narrow spectrum light	Broad spectrum light
Relative humidity 98-100%	Relative humidity 40-80%
Almost non-functional roots	Functional roots
Almost non-functional photosynthetic system	Functional photosynthetic system
Exogenous hormones	Endogenous hormones
Sterile conditions	Non-sterile conditions

Micropropagated plantlets should be acclimatized gradually by transferring them to a clean area under partial shade. In a low-cost hardening system, the culture containers are kept in the greenhouse with lids loosened. The plants are allowed to grow for 4-6 weeks under conventional Grow lights ^{TM}. This semi-hardens plants, and leads to shoot elongation, which is usually done under laboratory conditions. Contamination can be controlled with regular sprays of fungicides and bactericides. The plants are periodically sprayed with water to maintain humidity. Bench lamps are used to provide light, if and when required. Semi-hardened elongated shoot clumps are then separated under non-sterile conditions, and rooted in peat or plugs under thin plastic sheets for 2-3 weeks. This low-cost alternative reduces the capital cost of hardening by saving laboratory space, labour, and time. This procedure has been used on a commercial scale for a number of plants such as *Gerbera*, *Spathiphyllum*, *Syngonium*, African violets, *Chrysanthemum*, *Ficus*, *Philodendron* and other ornamentals in the Netherlands and Israel (Prakash and Peirik, 1991; Prakash, 1996). The growers in Florida harden tissue-cultured foliage plants in cavity trays covered with a transparent plastic tray, thus saving costs of a greenhouse. In some parts of India, hanging the culture plastic bags in shade hardens tissue-cultured sugarcane plants. Plants produced in this manner are semi-hardened, and can be delivered to the growers at a relatively cheaper price.

Low-cost plastic tunnels (polytunnel) with bio-fertilization can also be used for hardening, especially in cooler climates. A rectangular pit of desired and manageable size is dug on a site free of water logging. A frame of bamboo or any other available material is made above the pit with a gentle slope to one side. The frame is then covered with a clear polythene sheet, and tied using nylon ropes. The sheet is sealed with mud on three sides, leaving one side free. The temperature and humidity inside these structures is generally higher than the outside, and protects the plants from frost. The polythene sheet can be partly opened to allow air circulation and sunlight as and when required. The added advantage of polypits is the carbon dioxide fertilization effect and reduced need for watering. The International Institute for Tropical Agriculture, Ibadan, Nigeria has developed another low cost hardening alternative. The institute provides the farmers with plants in plastic bags and handling instructions. For hardening the plants, the farmers hang the plastic bags under tree shade.

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INTEGRATION OF TECHNOLOGY FROM LAB TO LAND

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Abstract. The price of the tissue culture derived plants, tubers, bulbs, and cuttings must be competitive with those obtained from conventional propagation. Many tissue-cultured plants are too expensive for direct field planting. In such cases, the cost can be reduced by one or more times conventional propagation of the tissue cultured plants. The uniformity and consistency in field performance of tissue-cultured plants is important to build confidence of the farmers to integrate such plants in the production systems

INTRODUCTION

Tissue culture technology contributes significantly to the improvement of agricultural productivity and food security. However, it must be integrated into production systems in a cost effective manner. Wherever practical, direct use of the tissue-cultured plants is the best strategy to obtain the full benefits of this technology. The price of the products, i.e. plants, tubers, bulbs and cuttings, must be competitive with those obtained from conventional propagation. While many plants that are propagated from vegetative parts can be micropropagated, the cost of tissue-cultured plants is still too high for direct growing by the farmers. For example, micro- and mini-potato tubers and micropropagated plants of chrysanthemum, strawberry, sugarcane and cassava are too expensive for direct field planting. In such cases, tissue-cultured plants are grown as super-elite or elite material and the costs are brought down by conventional propagation one or more times. Thus, the costly tissue-cultured plants are used as nuclear material, and their further multiplication is by conventional vegetative propagation in the field to produce large quantities of pathogen-free planting material. However, the subsequent vegetative multiplication of tissue culture-derived material in the open environment also exposes them to infection with diseases and viruses that may increase in successive propagation. In addition, the handling of bulky conventional planting material, such as potato tubers, banana corms and sugarcane sets adds to the transportation costs. Hence, the cost of tissue-cultured plants has to be brought down substantially, so that farmers benefit through increased productivity.

On-farm technology transfer

The most outstanding application of tissue culture in crops that are propagated from vegetative parts has been the production of disease-free planting material that gives improved yield and quality. This has been particularly the case in potato, banana, citrus, date palm, pineapple, papaya and many ornamentals. Seed potato production is a highly specialized business, and requires highly skilled growers. Currently, all the certified seed potato in Ireland and Scotland is derived from tissue-cultured plants, which produce micro- or mini-tubers that form the super-elite seed. The mini-seed tubers are not used for growing the potato crop. Instead, these are further multiplied by conventional propagation in the field, at least twice, and sometimes even 3 to 4 times for the production of certified seed tubers. Apart from cost reduction and high seed quality, the farmers get the same types of seed tubers with which they are used to. In Canada, many growers have integrated small tissue-culture laboratories for seed potato production on their farms (Nowak, J. *Personal communication*). Similarly, the sugarcane growers in Punjab, India have switched to high quality seed cane that is derived

from tissue cultured plants, which are propagated 3 to 4 times from conventional cuttings. The lead in commercial sugarcane tissue culture technology has been taken by the sugar cane mills, which have established their own laboratories for large-scale seed cane production. The state agricultural university provides the disease-free indexed cultures to the sugar mills. Such a model that includes the university for research and trouble-shooting problems, private industry (sugar mills) that multiplies the sugarcane seed material through tissue culture, and cane growers who produce the crop, has been very successful in integrating tissue culture technology for the benefit of the farmers and processors. This tripartite cooperation has rapidly replaced conventional sugarcane seed production in the state. Recently, direct planting of micropropagated sugarcane plants has been profitably undertaken in Maharashtra, India.

In Morocco, citrus indexed scions are multiplied in tissue culture and grown under a plastic house. Scion cuttings with two-three buds each are then sold to the farmers, who graft them on either new or old rootstock. This has helped to eradicate many of the citrus viruses and yet retain the old rootstock plants that are resistant to diseases. In another approach, high quality disease-free tissue-cultured scions are grafted *in vitro* onto seedling rootstock. Millions of such *in vitro* grafted plants have been distributed to the citrus growers in Spain, bringing direct benefits to farmers. In Thailand, tissue-cultured chrysanthemum plants, produced by the Department of Agriculture, are sold to farmers who multiply them further from conventional cuttings for the production of cut flowers. This procedure has allowed rapid introduction of new varieties to respond to the market demand. In Vietnam and China (P. R.), *in vitro* cultured plants of sweet potato and cassava are distributed directly to the farmers. The farmers multiply *in vitro* plants further from conventional cuttings, and produce sufficient amounts of healthy planting material for their on- farm use. A similar procedure could be used for the distribution of disease-free material of cassava in Africa where cassava mosaic virus disease has become a major threat to cassava production.

Perhaps, the most successful on-farm use of tissue culture technology has been in Cuba where an ordinary village-house is converted into a plant tissue culture facility – a 'bio-factory'. Such a facility employs local labor, particularly housewives, for various operations, uses low cost media and containers and sunlight, and has the capacity to produce one million banana or sugarcane plants per year. In banana, the hardened plants are sold to the local banana growers. A batch of one thousand plants is kept in the nursery to monitor the quality. The farmers are asked to notify any off-type, which they may observe. This has not only given the farmers a confidence in the technology but has also resulted in the discovery of several improved variants among the tissue culture derived populations.

In developed countries, many commercial companies restrict tissue-culture propagation to the production of *in vitro* plants, which are used as nucleus or 'super elite' material, and are further multiplied two to three times before being handed over to the farmers as certified material. Other companies subcontract the production of fully-grown plants on a buy-back basis for further sale. This approach has been used in the propagation of many ornamental plants and shrubs in Belgium. In the developing countries, tissue culture must be seen as a complementary approach to conventional plant propagation, so that the rural job opportunities are not lost, but strengthened and further expanded. Hence, on-farm conventional multiplication of the tissue culture propagated material is the cheapest and the most sustainable method for cost reduction, and for the creation of farmer confidence in the technology. Of course, the agricultural extension workers and propagation companies must provide the necessary know-how to the farmers for handling and growing tissue cultured plants for further multiplication under high health status. Universities and experts should provide training to farmers and extension workers as being done in several countries.

On-farm performance

Even after a decade of introduction of millions of tissue culture plants, the market covered is less than 1% of the total cultivation under vegetatively propagated crops. Thus, there is a tremendous scope to use tissue-culture technology to provide growers with nuclear stocks of shrubs, fruit and forest trees. There is a need to micropropagate hundreds of native varieties and local cultivars of banana and plantain, cassava, citrus, date palm, ginger, potato, sweet potato, turmeric, yams, and local tropical fruits, flowers, herbs, medicinal plants and trees that are propagated from vegetative parts.

The consistency in field performance of tissue-cultured plants is extremely important. Farmer's participation is essential in achieving this goal (Bunders *et al*., 1996). Precision agricultural management, based on integrated and sustainable crop nutrient practices, is required to obtain high yields from quality planting material. In some cases, the problem of unsatisfactory field performance was traced to nutritional imbalance and poor soil conditions (Savangikar *et al*., 1999). The commercial micropropagation companies should encourage the availability and use of site-specific agronomic practices by the growers to ensure sustainable success. This would provide farmers the latest technology product with minimal risk. Thus, to achieve expansion of commercial micropropagation, the use of cost-effective technology and of locally adapted varieties and relevant agronomic practices is necessary.

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SUMMARY

Plant tissue culture refers to growing and multiplication of cells, tissues and organs of plants on defined solid or liquid media under aseptic and controlled environment. Micropropagation allows rapid production of high quality, disease-free and uniform planting material. The micropropagation of high quality planting material of ornamentals, and forest and fruit trees has created new opportunities in global trading for producers, farmers, and nursery owners, and for rural employment. The plants can be multiplied under a controlled environment anywhere irrespective of the season and weather on a year-round basis. However, micropropagation technology is more expensive than the conventional method of plant propagation. It is a capital-intensive industry, and in some cases the unit cost per plant becomes unaffordable. Hence, it is necessary to adopt strategies to reduce production cost and lower the cost per plant.

Plant micropropagation is primarily based on rapid proliferation of tiny stem cuttings, axillary buds, and to a limited extent of somatic embryos, cell clumps in suspension cultures and bioreactors. The cultured cells and tissue can take several pathways. The pathways that lead to the production of true-to-type plants in large numbers are the preferred ones for commercial multiplication. The process of micropropagation is usually divided into several stages i.e., pre-propagation, initiation of explants, subculture of explants for proliferation, shooting and rooting, and hardening. These stages are universally applicable in large-scale multiplication of plants. The delivery of hardened small micropropagated plants to growers and market also requires extra care.

Low-cost tissue culture technology is the adoption of practices and use of equipment to reduce the unit cost of micropropagule and plant production. Low cost options should lower the cost of production without compromising the quality of the micropropagules and plants. In low cost technology cost reduction is achieved by improving process efficiency and better utilization of resources. Low-cost tissue-culture technology is high priority in agriculture, horticulture, forestry, and floriculture of many developing countries for the production of suitably priced high quality planting material.

A number of low-cost alternatives can be used to simplify various operations and reduce the costs in a tissue culture facility. The physical components of a typical plant tissue culture facility include equipment and buildings with preparation room, transfer room, culture or growth room, hardening and weaning area, soil-growing area (greenhouses, plastic tunnels), packaging and shipping area, and related facilities such as an office, and a store for chemicals, containers and supplies. The size of the physical components of a tissue culture facility will vary according to its functional needs, *i.e.* the volume of production. Careful planning of a facility can make large savings both in the construction costs and day-to day operations in the facility. It is recommended that an existing facility should be visited to view the layout and operational needs before starting a new facility.

Proper choice of media and containers can reduce the cost of micropropagation. The composition of culture media used for proliferation has a tremendous influence on production costs. The type of culture vessel influences the efficiency of transfer during subculture and production of propagules per unit area. The replacement of expensive imported vessels with reusable glass jars and lids, alternatives to gelling agents, use of household sucrose, and some medium components can reduce costs of production. Bulk making of media and storage as deep frozen stocks also reduces labour costs.

Artificial lighting of cultures in the growth rooms is one of the most expensive and inefficient methods in tissue culture technology. Changing the method of illumination from artificial to natural light is a decisive low cost option in tissue culture. This reduces electricity and capital costs and also improves the plant quality. Maintaining *in vitro* cultures at a regulated temperature with air conditioners adds to the cost but does not contribute to specific plant quality. Many *in vitro* growing plants can tolerate wide fluctuations in temperature, and adapt better to field conditions than those grown under even temperature. Plants can also be hardened in open shade. Plants hardened under natural light are sturdy, and withstand transplantation better in the field. Production of plants based on tissue culture technology and their subsequent growing is a labour intensive system. Even in developing countries, where labour is relatively less costly, hiring expertise from established R&D laboratories reduces overhead costs.

Bioreactor-based propagation of plants can increase rate of multiplication and growth of cultures and reduce space, energy and labour requirements in commercial micropropagation. They can therefore be attractive to developing countries as regards new or expanding plant culture facilities, in combination with a conventional laboratory. Bioreactors provide more precise control of the plant growth gaseous exchange, illumination, medium agitation, temperature and pH than the conventional culture vessels. However, to be cost-effective, use of bioreactors requires indexed plant cultures, and attention to aseptic procedures during handling of plant material otherwise culture contamination leads to massive economic loss.

In micropropagation, the health status of the donor mother plant and of the plants multiplied from it are among the most critical factors, which determine the success of a tissue culture operation. The indexing of the mother plants for freedom from viral, bacterial, and fungal diseases is a normal procedure in large-scale plant propagation through tissue culture. Plants not originating from pathogen-tested material must be screened for the presence of viruses. Laboratories, which do not have in-house facilities to carry out plant indexing, should obtain their indexed stock plants from organizations such as Departments of Agriculture, agricultural universities or privately owned certified germplasm repositories that routinely produce such plant material. Batches of micropropagated plants should be tested for freedom from diseases either in-house or by other laboratories. ELISA has been the most effective method for virus and pathogen detection in plants. Polymerase chain reaction and nucleic acid hybridization are more sensitive than ELISA, and can detect pathogens in extremely low amounts.

Quality control is essential to assure production of high quality plants and to have end-users confidence. Quality standards require the establishment of suitable tests to maintain quality control. The choice of explant source, freedom of the donor plant from viruses, disease causing fungi, bacteria, viroids, phytoplasmas, vigour and conformity of the variety, and elimination of somaclones are critical for maintaining plant quality. Variety identification by proper labeling at all stages is essential to ensure varietal identity.

Tissue-cultured propagules are produced under a controlled environment. Such plantlets have small juvenile leaves with reduced photosynthetic capacity, and malfunctioning stomata. Priming for rooting, shooting, and improved photosynthesis can be achieved with growth regulators and adjustment to the growing conditions that affect the post-transplanting performance of the propagules. Vented closures with microbial filters facilitate gas exchange, reduce ethylene build-up that stunts plant growth, reduces leaf-size, and causes leaf drop in tissue culture containers. Plantlets produced under photo-autotrophic culture systems on media with or without sucrose but CO_2-enrichment, increased light intensity, good gas exchange and reduced humidity are more vigorous, have larger root-systems, and are less susceptible to

microbial contamination. Plants adapted gradually to the *ex-vitro* environment have improved survival upon transfer to soil. Plants in their natural environment are colonized with many bacteria, fungi, and mycorrhizae. *In-vitro* or *ex-vitro* biopriming of micropropagated plants with such organisms improves plant performance under stress environments, and consequently enhances yield.

Low cost options can generally be incorporated into the design of the building, laboratories, working areas, layout of equipment, lighting, heating and production planning to provide smooth and efficient operations. It is important to select several plants that provide options for production around the year to allow cash flow and optimal use of equipment and facilities. It is essential to maintain sufficient mother cultures, and limit the number of subcultures to avoid variation, and plan production and shipment according to the customer's demand. The price of the tissue culture derived plants, tubers, bulbs, and cuttings must be competitive with those obtained from conventional propagation. Many tissue-cultured plants are too expensive for direct field planting. In such cases, the cost can be reduced by one or more conventional propagations of the *in vitro* plants. The uniformity and consistency in field performance of tissue-cultured plants is important to build confidence of the farmers to integrate such plants in the production systems.

EQUIPMENT CHECKLIST

The streamlined operation of a tissue culture facility requires a specific layout of the laboratories and strategic location of the equipment (Fig.1-3). The needs for equipment vary widely with the type of culture system used and the capacity of the facility. A conventional tissue culture facility has the following equipment and consumables, some of which are optional. To make the operations smooth, the equipment should be located in areas where it is most frequently used.

Preparation room

1. Autoclave (e.g. 125 l capacity, swift lock, single phase, Astell Hearson, UK)
2. Water distillation (20 l reservoir, e.g. Model A4D Aquatron, Bibby, UK)
3. Double sink unit
4. Hot plate with magnetic stirrer (e.g. Autostir, Cabburn)
5. pH meter (e.g. Delta II, Ciba-Corning)
6. Weighing balance 1 to 200g (e.g. Oertling TP 41)
7. Weighing balance1.0-0001 g (e.g. GA 110-Ohaus)
8. Oven for drying glassware
9. Microwave oven (e.g. M715 Phillips)
10. Refrigerator
11. Freezer (optional depending on the lab requirement)
12. Trolley for carrying hot media flasks and containers
13. Cupboards along the walls for storage of chemicals and presses underneath for storage of glassware and containers.

Transfer room

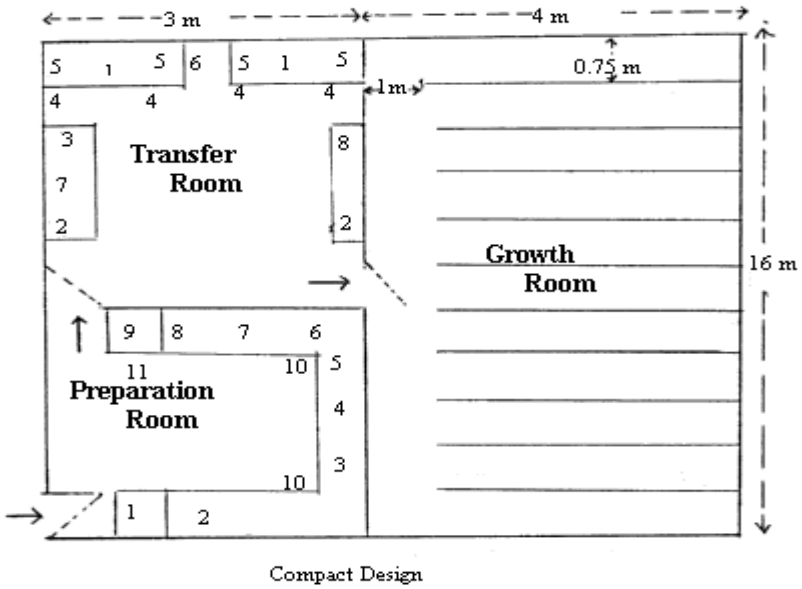
1. Laminar Flow cabinets (double bench) (e.g. Gelaire TC 72 or TC48
2. Bench with presses for storage of containers with media.
3. Peristaltic Pump for pouring medium (e.g. MKII, Accuramatic, UK)
4. Height adjustable chairs (computer chairs) or swivel chairs
5. Safety burners (e.g. Touch-a-Matic) and gas cylinder or preferably Glass bead sterilizers
6. Binocular microscope
7. Gyratory shaker (optional)
8. Inverted microscope (optional)
9. Compound microscope
10. Shaker for low speed use, meant to grow shoots and rooting in liquid medium

Growth room

It is desirable that the floors and walls in this room have white tiles. Floors can also be covered with linoleum instead of white ceramic tiles.

1. Shelving unit frames - each unit is 3.0 x 0.6 m with four tiers spaced 0.45 m apart; the first tier is 0.15 m above ground. Frames can be made from angle iron similar to that used in supermarket shelving. A corridor of 0.70-0.75 m should be left between the shelving units and 1.0 m in the front. The shelves are made of thick wire net or punched light iron sheets to allow air circulation. Shelving-unit frames can also be made of steel or wood.
2. For artificial lighting, use two 65W cool daylight fluorescent tubes per shelf (8 lights/unit), with chokes located outside the growth room. The time switches (timer clocks) should be located outside or just at the entrance.
3. Air-conditioning should be located inside and the compressor unit outside. Exhaust fans to expel hot air linked to reverse thermostats can be used as replacement to air-conditioners in milder climates, particularly if lighting is from diffused natural daylight.

Plan for a tissue culture facility



Preparation Room

1. Autoclave
2. Water distillation and sink unit
3. Hot plate stirrer
4. pH meter
5. Weighing balance
6. Weighing balance
7. Hot-air oven
8. Microwave
9. Refrigerator
10. Cupboards and presses
11. Trolley

Transfer Room

1. Laminar flow cabinets
2. Bench and presses
3. Peristaltic pump
4. Chairs
5. Safety burner/sterilizers
6. Gas cylinder
7. Binocular microscope
8. Gyratory shaker

FIG. 1. A sketch plan for setting up a Plant tissue culture laboratory.

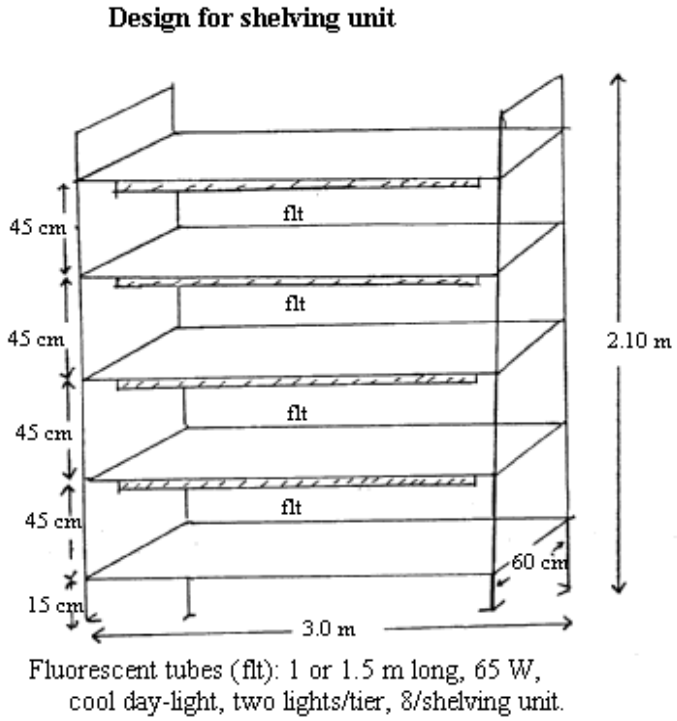


FIG. 2. Shelving unit made from angle iron.

4. Thermometer with maximum-minimum temperature recording
5. Light meter for measuring light intensity

Hardening and growing area
1. Shelves made of bamboo
2. Black plastic for flooring
3. Thatching for roofing the hut

Glasshouse
1. Heat dissipating nest (optional)
2. Plastic and pipes for weaning dome (optional)
3. Matting for the floors
4. Hose for watering plants or a watering can

Consumables
Chemicals for media and explant sterilization
Glassware – Duran bottles with rims (100, 250, 500, 1000 ml), glass cylinders (10, 50, 250, 500 1000 ml), beakers (25, 500, 1000 ml); Erlenmeyer flasks (250, 500, 2000, 3000 ml)
Surgical blades and knifes, forceps, and scissors with long handles
Pipettes (0.1, 0.5, 1.0, 5, 25, 50 and100 ml)
Buffers for pH meter
Containers- baby food jars, jam jars, other type of reusable vessels (e.g. Magenta ^{TM})
Nylon Ultra filters
Syringe (25, 100 ml)

Suppliers of equipment and chemicals*

Before ordering equipment, it is always good to look for the local supplier and seek advice from the nearest college or university on the prices, suppliers and alternative products and to search the Internet for suppliers.

J. Bibby Science Products Ltd., Stone, Staffordshire ST15OS, UK (water still)

Millipore, UK. Ltd., Boulevard Blackmoor Lane, Watford, Herts, WD1 8YW (filters)

Karir International Ltd. 17 Bishops Close, Mays Lane, Arkley, Barnet Herts EN5 2QH, UK (chemicals, media, tissue culture containers)

Schott Glaswerke, Hattenbergstrasse 10, 6500 Mainz, Germany (bottles and glassware)

Scholte Winschoten, b.v. J.A. Koningstraat 1, 9672 A Winschoten, The Netherlands (laboratory furniture)

Kord Products Inc. Brampton, ON, Canada (for K1020 plastic trays and covers for plant acclimatization) http://www.kord-specialty.com

Jiffy Products Ltd., Shippagan, NB, Canada (For Jiffy plugs)

Sigma, St Luis, MO, USA (for chemicals, media, culture vessels, vented closures)

Duchefa, Haarlem, Netherlands (for, chemicals, media, culture vessels, vented closures)

Temporary immersion bioreactor (developed by CIRAD- Vitropic SA, ZAE des Avants, 34270 Saint Mathieu de Tréviers, France http://www.cirad.fr/produits/rita/en/accueil.htm

Pre-sterilized bioreactor: LifeReactor™, Osmotek Ltd., Rehovot, Israel www.Osmotek.com

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*The mention of any make, model and supplier does not constitute a recommendation by FAO or the IAEA.

COMMONLY USED TERMS IN TISSUE CULTURE

Adventitious: development of organs such as buds, leaves, roots, shoots and somatic embryos from shoot and root tissues and callus.

Asepsis: without infection or contaminating microorganisms.

Aseptic technique: procedures used to prevent the introduction of microorganisms such as fungi, bacteria, viruses, and phytoplasmas into cell, tissue and organ cultures, and cross contamination of cultures.

Axenic culture: a culture without foreign or undesired life forms but may include the deliberate co-culture with different types of cells, tissues or organisms.

Callus: an unorganized mass of differentiated plant cells.

Cell culture: culture of cells or their maintenance *in vitro* including the culture of single cells.

Cell generation time: the interval between consecutive divisions of a cell.

Cell line: cells that originate from a primary culture at the time of the first successful subculture.

Chemically defined medium: a nutritive solution or substrate for culturing cells in which each component is specified.

Clonal propagation: asexual multiplication of plants from a single individual or explant.

Clones: a group of plants propagated from vegetative parts, which have been derived by repeated propagation from a single individual. Clones are considered to be genetically uniform.

Cryopreservation: ultra-low temperature storage of cells, tissues, embryos and seeds.

Differentiated: cultured cells that maintain all or much of the specialized structure and function typical of the cell type *in vivo*.

Diploid: cells, tissues and organisms, which have two sets of all chromosomes, except the sex chromosomes.

Embryo culture: *In vitro* culture of isolated mature or immature embryos.

In vitro: Latin: "in glass" - culture of an organism or a portion of it in glass or plastic ware on synthetic media.

Tissue culture: *in vitro* culture of cells, tissues, organs and plants under aseptic conditions on synthetic media.

Growth chamber: a chamber used for the incubation of culture containers or plants under controlled environment

Micropropagation: multiplication of plants from vegetative parts by using tissue culture.

Propagule: a portion of an organism (shoot, leaf, callus, etc.) used for propagation.

Explant: an excised piece or part of a plant used to initiate a tissue culture.

Subculture: the aseptic division and transfer of a culture or portion of that culture to a fresh nutrient medium.

Meristem: a group of undifferentiated cells situated at the tips of shoots, buds and roots, which divide actively and give rise to tissue and organs.

Somatic embryos: non-zygotic bipolar embryo-like structures obtained from somatic cells.

Totipotencity: capacity of plant cells to regenerate whole plants when cultured on appropriate media.

Transgenic: plants that have a piece of foreign DNA.

ABBREVIATIONS

ABA	Abscisic acid
BA	6-benzyladenine
BAP	6-benylaminopurine
BAR	6-benzylaminopurine riboside
BPA	N-benzyl-9-(2-Tetrahydropyranyl) adenine
4-CPA	p-chlorophenoxyacetic acid
2,4-D	2,4-dichlorophenoxyacetic acid
2,4,5-T	2,4, 5 -trichlorophenoxyacetic acid
DMF	Dimethylformamide
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
2iP	6-(-y,-rdimethylallylamino) purine
2iP-R	6-(y,-rdimethylallylamino) purine riboside
GA3	Gibberellic acid
IAA	Indole-3-acetic acid
IBA	lndole-3-butyric acid
KIBA	Indole-3-butyric acid-potassium salt
IPA	lndole-3-propionic acid
KR	Kinetin riboside
MES	2-[N-morpholino] ethanesulfonic acid
NAA	α-Naphthaleneacetic acid
NOA	o-Naphthoxyacetic acid
ZR	Zeatin riboside

Table 1. Composition of commonly used tissue culture media (mg/l)

Components	Murashige and Skoog, 1962	Gamborg *et al*., 1968	White, 1963	Lloyd and McCown, 1981	Vacin and Went, 1949	Modified Knudson, 1946	Mitra *et al*., 1976	Nitsch and Nitsch, 1969
Macronutrients								
$Ca(PO_4)2$					200			
NH_4NO_3	1650			400				720
KNO_3	1900	2500	80		525	180	180	950
$CaCl_2 2H_2O$	440	150		96				166
$MgSO_4 7H_2O$	370	250	720	370	250	250	250	185
KH_2PO_4	170			170	250	150	150	68
$(NH_4)_2SO_4$		134			500	100	100	
$NaH_2PO_4H_2O$		150	16.50					
$Ca(NO_3)_2 4H2O$			300	556		200	200	
Na_2SO_4			200					
KCl			65					
K_2SO_4				990				
Micronutrients								
KI	0.83	0.75	0.75			80	0.03	
H_3BO_3	6.2	3	1.5	6.2		6.2	0.6	10
$MnSO_4 4H_2O$	22.3		7		0.75	0.075		25
$MnSO_4H_2O$		10		29.43				
$ZnSO_4 7H_2O$	8.6	2	2.6	8.6			0.05	10
$Na2Mo0_4 2H_2O$	0.25	0.25		0.25		0.25	0.05	0.25
$CuSO_4 5H_2O$	0.025	0.025		0.25		0.025	0.05	0.025
$CoCl_2 6H2O$	0.025	0.025				0.025		
$Co(NO_3)_2 6H_2O$							0.05	
Na_2EDTA	37.3	37.3		37.3		74.6	37.3	37.3
$FeSO_4 7H_2O$	27.8	27.8		27.8		25	27.8	27.8
$MnCl_2$						3.9	0.4	
$FeCC_4H_4O_6)_3 2H_2O$					28			
Vitamins and other supplements								
Inositol	100	100		100				100
Glycine	2	2	3	2				2
Thiamine HCl	0.1	10	0.1	1		0.3	0.3	0.5
Pyridoxine HCl	0.5		0.1	0.5		0.3	0.3	0.5
Nicotinic acid	0.5		0.5	0.5			1.25	5
Ca-panthothenate			1					
Cysteine HCl			1					
Riboflavin						0.3	0.05	
Biotin							0.05	0.05
Folic acid							0.3	0.5

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