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COMMERCIAL ASPECTS AND PROBLEMS IN MICROPROPAGATION

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INTRODUCTION

Biotechnology, which can be broadly defined as the commercialization of biology via cell, organ and tissue culture, and recombinant DNA technology has made giant strides during the past 20 years and expected to contribute significantly to solving four global issues, namely, a shortage of food, a shortage of fuel energy and/or natural resources, environmental pollution and destruction of ecosystems. These issues will become increasingly critical in the early 21st century, when the world population is predicted to nearly 10.8 billion by 2050.

Biotechnology has rapidly been made big business and big science. Evidence of this can be seen in the scores of biotechnology parks and research institutes, hundreds of biotechnology companies, uncountable conference and symposiums, and giant research programs like the human genome project, that have been established during the relatively short period of 20 years since 1975.

Plant biotechnology, like its biomedical counterpart, has two important but critical interacting technical components, those of tissue culture and molecular biology. Interest and investment in plant molecular biology is rather recent but is already paying diverse remarkable results in the understanding of the molecular basis of plant growth and development (22,52). Cell culture techniques have been available and continuously improved since the early 1930's (18) and have been use for a long time. Many of the procedures used are simple and can be practiced without substantial investment in facilities and infrastructure for the production and multiplication of improved plants. These constitute the short term and/or immediate application of this technology such as superior clonal propagation, elimination of viruses and diseases from many plants, embryo rescue of young hybrid embryos, haploid production via anther or microspore culture, and production of secondary metabolites using bioreactors. The developing technologies which are based on the integration of cell culture and molecular genetic techniques for limited but imminent plant improvement provide a variety of intermediate term applications. Major impact of plant biotechnology on agricultural production must await significant scientific advances in the understanding of plant growth and development for long-term applications beyond the year 2000.

HISTORICAL BACKGROUND

Orchid micropropagation was the key event in the founding of the micropropagation industry (Morel, 1974). Culture of meristem-tips yielded protocorms that could be cut up and recultured. The rate of multiplication was far more rapid than other vegetative means of propagation and the aseptic techniques required were already familiar to those producers germinating orchids seed in vitro. At the time, it was not clearly understood by many practioners of micropropagation

that using tissue culture methods did not automatically insure that the plants produced would be virus free. Thus, an unintended consequence of the early production, particularly by small laboratories, was the spread of virus-infected orchids, rather than the production of disease-free plants.

The meristem-tip culture technique, originally shown to be effective for *Dahlia* and potato (Morel and Martin, 1952, 1955), was applied to numerous other crops. The techniques developed in this research, together with the work on micropropagation of orchids and the development of a widely adaptable tissue culture medium (Murashige and Skoog, 1962), led to successful *in vitro* propagation of numerous crops.

Most of the early commercial applications of micropropagation were to foliage plants widely used in the rapidly expanding area of interior landscaping (25). Considerable success was also achieved with flower crops of various types (Oglevee-O'Donovan, 1986. Paek and Thorpe, 1990a; Stimart, 1986)

Many researchers contributed to the efforts to adapt micropropagation technology to woody plants. A key contribution was the report by Jones (1976) that phloridzin and phloroglucinol would stimulate axillary shoot production on apple shoot tips *in vitro*. This information spurred much research on apple and other woody plants, which led to the development of protocols successful for numerous species and cultivars.

The use of micropropagation has been limited to relatively few vegetable crops, but it is advantageous in certain situations, particularly for asparagus, potato and sweet potato. It is currently applied on a large scale to production of certified potato plants to be used for minituber production of certified potato plants to be used for minituber production in the greenhouse or seed tuber production in the field. The technology has been reviewed by Jones (1988).

The growth of commercial micropropagation has not been steady. Early successes with many crops through the early 1980s led to a rapid increase in number and size of commercial laboratories by 1985. This quickly resulted in overproduction of certain crops and economic competition became very severe. Some laboratories closed and others consolidated within the industry. Production once again seems to be increasing as demand continues to grow.

Newly developed protocols continue to broaden the range of plants for which it is economic to use micropropagation on a commercial scale. Efforts with forest tree species have lagged because of two reasons: the species of interest are often recalcitrant in culture and the cost per propagule must be very low to make the method economically feasible. Nevertheless., significant progress has been made and small scale production trials can be anticipated in the near future.

BENEFITS OF MICROPROPAGATION

Several distinct advantages are associated with the commercial production of plants by micropropagation, and man authors have discussed them (20,39,40,41,49). From a commercial perspective, the advantages of micropropagation may best be organized into three important areas in the development and marketing of an improved product: (1) product development, (2) product enhancement, and (3) marketability of product.

Rapid Multiplication. The ability to increase plant material rapidly can favorably impact the development and release of both asexually propagated varieties, generally produced by cuttings, and sexually propagated varieties, produced by seed. In both cases, micropropagation

offers a method to increase valuable genotypes rapidly and expedite the release of improved varieties. In addition, there is a significant reduction in required stock plant numbers and necessary growing space for both asexually and sexually propagated crops. In the case of asexually propagated crops, micropropagation could be used both to rapidly increase new selections and to produce the ultimate product. In the case of sexually or seed-propagated crops, valuable breeding lines and/or parental lines used in hybrid seed production could be rapidly increased (2). Of particular interest would be the increase of male sterile lines, whose maintenance and increase normally require much time and backcrossing. Several major seed companies have recognized the value of this aspect of micropropagation, particularly for vegetables and ornamentals (33). It has been estimated that, by the rapid increase of parental lines, a hybrid variety could be released 3 or even 5 years sooner, compared to the normal time required by utilizing stock seed increase of parent lines.

Product Uniformity. Especially in the case of product development, micropropagation methods which maintain and ensure the genetic integrity of the initial material are utilized. Besides genetic or clonal uniformity, a high degree of phenotypic uniformity is possible as well. Since a large portion of the production cycle takes place under artificial conditions and crop scheduling is easier to control, the resulting product has a very high degree of uniformity as compared with that produced by other propagation methods. Plants produced and marketed as plugs, especially, represent a uniform, easy-to-handle product.

High Volume. Micropropagation allows for the production of large numbers of plants in a relatively smaller space or growing area and in a relatively shorter time. This benefit is particularly valuable in the case of product development and the release of new varieties. For hybrid varieties, large populations of parent lines are required. In addition, the timely release of a new asexually propagated variety requires very large numbers to supply its demand and establish a strong market standing. Thus, even if only one plant of a desirable genotype exists, it would be possible to clonally propagate as many plants as required. This application will be particularly valuable in the improvement of crops with long growing periods, such as trees.

Heterozygous Products. Micropropagation techniques provide the opportunity for reproducing a phenotypically uniform plant population from genetically heterozygous plant material. For asexually or vegetatively propagated material, such as woody ornamentals, this application is not novel. However, its use in the development and release of varieties of crops conventionally seed-propagated is a newer concept. In the production of hybrid seed, two inbred or homozygous parent plants are required for sexual hybridization. By tissue culture micropropagation, it is possible to clonally propagate a desirable heterozygous plant and actually bring it into commercialization. In this particular application, the time required for breeding and product development is greatly reduced because inbreeds are not required. In addition, any potential problems associated with inbreeding depression are circumvented. This technique could find particular application with crops such as celery, hybrids of which are difficult to produce.

Genetically Engineered Products. Micropropagation will be the major method of bringing improved plant varieties resulting from gene transfer programs into commercialization. Because plants resulting from cell fusions or gene transfers are often heterozygous or even sterile, micropropagation techniques will be useful to increase their numbers and expedite their eventual release.

Germplasm Storage. The ability to preserve and store germplasm can be of great value in the development of new products (20,43). Storage methods include those designed for short periods, of a few to several weeks, to those appropriate for longer periods, even years. Short-term methods include cold storage, minimal growth culture media containing elevated sucrose concentrations or growth retardants (20), and low partial pressures of oxygen (4). Longer-term methods would include cryopreservation or freezing in liquid nitrogen (16).

Short-term storage is particularly useful for plant-breeding applications. Selections may be held in storage until they are required in larger numbers for use in seed production, thus allowing for a times increase. Obviously, this technique is valuable for the storage of parental lines used for hybrid seed production, particularly when male sterile and maintainer lines are involved. In addition, species or varieties with seed viability or low germination percentages could also be stored *in vitro* for future use. When large populations of selected breeding or parental lines are required, they can be increased rapidly on short notice of cultures are already in storage. Since the cultures have already been successfully initiated and maintained, valuable time in the production schedule is saved and mass propagation, with an even shorter target date, is expedited.

Improved Phenotype. The resulting phenotype of the micropropagated plant can be controlled by the *in vitro* culture process. Characters that can be controlled by the growth hormones and regulators utilized in the culture medium include growth habit, such as basal branching; higher growth rate; faster flowering and/or fruiting; and enhanced color and quality. In particular, a well-branched growth habit with a fuller appearance is often cited as a desirable characteristic appreciated by commercial growers. For example, micropropagated *Syngonium* is characterized by a greater degree of basal branching than that produced by conventional stem-tip cuttings. It is generally thought that this increased branching is due to a "hormone carryover effect" typical of cytokinins, which can cause increased branching when applied to the whole plant. Other crops which benefit in this regard include *Spatiphyllum*, *Anthurium*, and *Ficus*.

Disease-Free Plants. Micropropagation provides the means of incorporating techniques used to free a plant of specific diseases. In general, the aseptic requirement of the tissue culture process results in the production of plants that are cleaner, especially regarding bacteria and fungi. To illustrate, micropropagated *Gypsophila* has become popular with cut flower producers (10). *Gypsophila* is often infected with crown gall, which is difficult to eradicate because it is transmitted through conventional vegetative propagation. Micropropagation allows for the selection and rapid increase of healthy material. For that reason, *Gypsophila* is becoming an important tissue culture product.

It must be understood, however, that the micropropagation process itself does not guarantee the removal of specific pathogens. Specific techniques, such as meristem culture and disease indexing, must be incorporated in the micropropagation process to provide the necessary conditions to eradicate a specific pathogene. These techniques have been incorporated into commercial micropropagation schemes to produce high-health ornamentals such as geranium, carnation, and chrysanthemum, as well as to produce such food crops as potato and strawberry (29). The added advantage is that, once a plant culture is established and determined to be free of a specific pathogene, micropropagation provides the capability to both maintain pathogen-free status and rapidly

produce the required number of plants upon demand.

Product Format. The micropropagation system allows for a diverse range of products for the grower, including plants fully established in soil, unrooted shoots or microcutting, and cultured clumps of shoots. Depending on a grower's facilities and experience in handling *in vitro* material, most growers prefer to purchase small starter plants which are fully acclimated and established in soil (47). Most micropropagation companies concentrate on this type of product, which is commonly produced as a plug in a tray format.

Movement of Product. The potential to produce material certified free of particular pathogens allows for greater ease in exchange of plant material between different countries (28). The transfer of plant material in the *in vitro* condition allows for transfer free of soil, which greatly speeds inspection at international entry points. An increasing quantity of plants are being shipped *in vitro*, whether as germplasm, production materials, or product. However, it should be realized that plants known to be carriers of specific viruses are still banned by some countries, whether they are in the in vitro state or not. Another aspect of ease of international exchange of plant tissue cultures is the potential utilization of offshore production to capitalize on lower labor costs and expanded markets. Thus, plant tissue cultures could be produced to a given stage offshore and then be returned to the main production facility, or even the customer, for finishing.

Nonseasonal Production. Since the plants are produced in an artificial environment, production can occur year-round and thus serve a more diverse market area. This potential is especially valuable when one considers international markets, particularly the reversed seasons of the northern and southern hemispheres. Thus, micropropagation could be carried out year-round, even 24 hours a day, and lead to highly efficient, cost-effective production. In addition, since the production process is theoretically very controlled, it can be accurately planned. Thus once the culture requirements, multiplication rates, and procedures for acclimation and establishment in soil are defined for a given crop, production cycles can be scheduled to meet peak demands. In addition, the ability to cold-store cultures further contributes to defining production schedules.

LIMITATIONS OF MICROPROPAGATION

Although numerous advantages and benefits are associated with tissue culture micropropagation, there are three major limitations to the largescale commercial application of the technology: (1) product line limitations, (2) customer acceptance, and (3) high production costs. With concerted effort over the past decade, these three limiting factors have been reduced in contrast to the initial introduction of micropropagation into the horticulture industry in the early 1970s. At that time, tissue culture was regarded as a grower's panacea and the limitations of the state of the technology were poorly understood (10). Problems with crop scheduling, inconsistent quality, seasonality, and micropropagation protocols which had been adequately tested were then common and serious. The grower's knowledge of the handling of tissue culture products was lacking, and large losses resulted. In addition, initial clonal micropropagation efforts led to the spread of serious plant pathogens, particularly orchid viruses (Lawson and Hearon, 1973). The spread of bacterial and fungal pathogens, such as *Xanthomonas* and *Erwinia*, were observed in the foliage plant industry (27,32). Tissue culture production companies must routinely incorporate culture indexing procedures during the culture

initiation period as well as later in the production cycle to be able to monitor and control any spread of pathogens or contaminants.

Production Protocol. Currently, the choice of crops to be produced by micropropagation is limited to the species for which acceptable micropropagation protocols have been defined. Second, the product line is further determined by the market demand (20). Although there are numerous reports in the literature of micropropagation systems for a wide range of plant species, they are often not amenable to scale-up into a commercial level of production (21). To be commercially feasible, a successful production protocol must be characterized as being highly reproducible and having acceptable procedures for culture initiation, rapid multiplication, maintenance of a high multiplication rate upon numerous subcultures or generations, rooting, acclimation, and establishment in soil. When the literature for a given species and its applicability to commercial micropropagation is reviewed, one or more of the mentioned requirements often cannot be met without further research and development. An increasing number of commercial micropropagation companies are expanding their own in-house research efforts because of their recognition of this fact (42). Furthermore, the market demand must be analyzed to determine which products merit the added expense of system development.

Product Quality. The ability to deliver a product with consistently high quality is of prime importance and directly impacts customer acceptance of micropropagated plants. That is particularly true of ornamental plant species, such as foliage plants, of which every portion of the plant will ultimately be evaluated for its quality and thus marketable value (25).

To ensure a high level of quality, it is very important to direct particular effort toward quality control, including reducing of off-types, grading by size, and product trials or grow-outs, if possible. Particular concerns include both phenotypic and genetic stability of the final product. In has been well documented in the literature that phenotypically or genetically variant plants can arise in culture, depending on the associated culture media, explant source, and plant regeneration system utilized (34). Careful monitoring of such factors as the culture media (particularly types and concentrations of growth regulators), avoidance of a callus intermediate and/or adventitious shoot production system, a short subculture time interval, and a short total duration in culture, can help minimize epigenetic, short-term developmental changes and permanent genetic changes (29).

Product Delivery. The ability to schedule crops accurately and deliver a defined quantity of product consistently are important factors in customer acceptance. Micropropagation companies range in their emphasis on contractual vs. free-sale production. Currently, micropropagation is best suited to provide a steady stream of plant material rather than to adapt to a customer's seasonal requirements (24). Since most commercial labs require a purchase commitment from the customer of up to 1 year and a notice of at least 3 months prior to changes in requested quantities, long-range planning is necessary for both purchaser and producer of micropropagated plants (24). In addition, familiarity with cultural procedures required for successful establishment of tissue-cultured plants is necessary, especially if the plants are not already fully acclimated to in vivo conditions and established in soil. Special procedures must be followed if plants or shoots are purchased while still grown *in vitro* (24). Careful attention to these recommendations is essential to the successful transfer and ultimate grower acceptance of *in vitro* products.

PROBLEMS IN MICROPROPAGATION

Contamination. Tissue culture contaminants, even those that are subliminal, may cause economic losses for the micropropagator, by overrunning the culture either killing the explant or rendering it unfit for subculture. This problem is exacerbated where the contaminant is not expressed during early stages to be expressed downstream in production. Secondly, subliminal contaminants may affect the productivity both *in vitro* and of the progeny plants (36,38). Included in the non-expressed or subliminal contaminants may be latent pathogens of the crop or of other crops. Both classer may cause economic losses when the microplants are exposed to different nutrient regimes or environments (26). Economic losses depend on the type of business the micropropagator is in (30), viz, supply of *in vitro* cultures to external nurseries for growing-on or growing-on in-house. Those trading internationally in *in vitro* cultures are particularly at risk where phytosanitary inspection is involved and a single contaminated culture detected may result in the destruction of the whole shipment. In traded cultures, microplants etc., where health status is certified, customers may take legal action and claim consequential loss for poor performance.

For all of the foregoing reasons, the micropropagator must exercise quality control over production (8). The basis of the phytopathological aspects of quality control are: 1) Awareness of the range and natural history of possible contaminants of the crop, including specific pathogens (51). 2) Adequate preparation of the donor plant (11) including treatments to reduce or eliminate pathogens and promiscuous endophytes (7) based on sound screening techniques. 3) Confirmation of the axenic (contaminant-free) status of cultures in Stage 1 (or prior to mass clonal multiplication) following employment of strategies to obtain healthy cultures and again based on reliable screening methods. 4) Rigorous monitoring of production to confirm the axenic status of the cultures. In large-scale production this will necessitate sampling production and is dependent on an appropriate sampling protocol (9). 5) An awareness that the spectrum of contamination microorganisms may alter with time in culture. This is a reflection of a shift in the origin of contamination from those associated with the donor plant to those inhabiting the laboratory including the staff (37). Contamination introduced by mites, etc. may also occur (3). 6) Monitoring of progeny based on sampling of production. This may be carried out in association with testing of the genetic stability of production (8). The underlying concern is that latent organisms may be clonally present below the level of detection. These may only be expressed or detectable in maturing or mature tissues of the progeny plant.

Screening of Tissue Culture and Regenerated Plants

Stage 1 cultures. Contamination in Stage 1 originates from the explant and can overrun the medium and thus be visually detected or pass subliminally with the risk of downstream expression. The issue of carry over of inoculum on explant surfaces and the explant preparation may be inadequate (15,20). Where the explant appears uncontaminated, it may be necessary to bulk up the material to provide adequate material for testing. This may involve transfer to Stage 2 media. Low titre of contaminants may frustrate detection in sap extracts of primary explants due to the inhibitory action of plant products. This problem may be overcome in the bulking up phase. Contaminant enrichment may also be achieved by culturing tissue sections, rather than

sap extracts, on bacteriological media (9).

The screening should include the two standard elements. Firstly, testing for cultivable organisms, and secondly, screening for known pathogens of the crop. Only cultures that are negative in both respects should be clonally multiplied in Stage 2. Contaminated cultures should be killed by autoclaving, etc., before being disposed of to avoid contamination of the laboratory.

Stage 2 cultures. It is recommended that only axenic cultures are proliferated in Stage 2. It is arguable, however, whether axenic status can be achieved for all crops at an economic cost in commercial micropropagation. It should be appreciated that the magnitude of this problem depends on the origin of the plant, knowledge of its pathology and the availability of screening procedures.

If the recommendation that only axenic cultures are proliferated in Stage 2 is followed, then any contamination arising in Stage 2 will come from faulty techniques or procedures. The main sources are instruments, where yeast may pass through alcohol sterilization procedure, hear resistant spores, e.g. *Bacillus*, which may pass through flame sterilization, or contamination by bacteria, etc., which may come from the micropropagator. Microorganisms may also enter the system through failure of the laminar flow hood, incompletely sterilized media or equipment. Contamination may also occur in the growth rooms where exchange is possible between the interior of the culture vessel and the external atmosphere. The latter may be avoided by the use of scaled gas permeable containers (8).

Leifert et al., (1989b) have monitored the range of cultivable bacteria isolated from cultures over time and have provided valuable confirmation of this trend noting a drift with time from plant-associated bacteria to environmental human-associated bacteria. Those screening for cultivable microorganisms should be aware of this change in the spectrum of contaminants.

Stage 3 cultures. To satisfy quality control standards, all production stages should be monitored as for Stage 2 production. Equally importantly, progeny plants should be screened particularly for known pathogens of the crop that may, below the level of detection, have been clonally transmitted in production. To date there has been little published on studies on the detection in tissue cultures of intracellular microorganisms. Gallenberg and Jones (1985) have reported the detection of potato viruses in potato nodal cultures. Further investigations in this area are urgently required to confirm the generality of Gallenberg and Jones' findings. A proposed integrated screening strategy is presented in Table 1.

Table 1. Screening for pathogens and contamination organisms in micropropagation.

Stage	Action
0	Visually examine potential donor plants for disease symptoms, screen for cultivable bacteria, use streak plating to separate isolates, use serial dilution to determine contamination. Use specific tests for known pathogens of the crop. Reject heavily contaminated or diseased individuals if practical.
I	Visually examine cultures for contamination, including "halo" formation. Reject all contaminated cultures. Screen remaining cultures for cultivable organisms and carry out specific tests for known pathogens of the crop. Reject all infected cultures.
II	Monitor production: sample production, examine visually and carry out tests for cultivable organisms. If contamination is detected screen upstream and downstream production, reject as appropriate.
III	The same manner as stage II.
IV	Established progeny should also be sampled and monitored principally for known diseases of the crop which may have been below the level of detection in vitro.

Hyperhydricty. Herbaceous and woody plants propagated *in vitro* are frequently affected by the redundant presence of various culture factors that lead to metabolic and morphological derangements. Anatomical, morphological and physiological anomalies in tissue cultured plants have been described by diverse terminology - hyperhydricity, vitrification, translucency, hyperhydration, succulency and glassiness. Although vitrification is an misused term because it refers to a physical and not a biological process, it is the most often used one, since in describes changes in the leaves, giving rise to a glassy appearance. Some of these features can be seen quite clearly in carnation leaves. The disorders (13), which are manifested mainly in the leaves, affect the two major processes carried out by the leaves, namely photosynthesis and gas exchange (CO_2, H_2O vapor). Anomalous anatomy is manifested to a lesser extent also in the stems and roots. These disorders in turn impede micropropagated plant establishment ex vitro (44,46)

The special requirements for shoot proliferation *in vitro* - high humidity, superfluous nutritional factors, both minerals and carbohydrates, high levels of growth regulators and low light intensity, are the major causes found to induce shoot malformation (19, 59). Recent evidence (12) indicates that the relative humidity and the water potential are the key factors involved in abnormal morphogenesis *in vitro*.

Characteristics of vitrified plants. The complex nature of abnormal morphogenesis *in vitro* and the resemblance of some of the physiological events to processes occurring in plants under stress (19), emphasize the need for the optimization of plant culture conditions *in vitro* (12,53). Although the various factors involved in the development of vitreous plants were studied, the relationship among them is still unclear. It appears that the various manifestations of hyperhydricity as a result of the effects of culture conditions are not through one key process but can occur separately or concomitantly. The different anatomical and physiological defects are the result of various disorders in metabolic pathways (44). Changes in protein synthesis affect various enzymes related to photosynthesis (Rubisco), to cellulose and lignin synthesis (glucan synthase, PAL), or to processes associated with ethylene production (peroxidases). These changes in proteins synthesis are thus effecting interrelated metabolic pathways. Protein levels were lower in vitreous leaves and a 30 kD protein was found to be present in vitreous but not in normal leaves (61). Peroxidases associated with lignin synthesis, which were shown previously to be related to lignification (Kevers et al., 1984) were found to have a MW of 30-32 kD (57).

The malformation and malfunctioning of plants are affected through several steps that are finally manifested in plant vitrescence. Morphologically and physiologically abnormal plants cannot survive ex vitro stress after transplanting; therefore they require a gradual transition period to acquire normal morphology enabling their survival. In several herbaceous species, persisting *in vitro* features in organs formed before transplanting cannot be repaired. Newly formed organs developing under *in vivo* environmental conditions will assume normal function. In apple and other woody species, *in vitro* formed leaves can survive transplanting, but do not expand to full size, stage, contributed to the development of normal transplantable plants (5). A different solution could be found through the use of growth retardants that reduce or inhibit leaf development. In gladiolus (60) and *Philodendron* (1), growth retardants, such as ancymidol and paclobutrazol, reduced leaf expansion and enhanced bud or corm proliferation. In *Philodendron* it was shown that the presence of 1.5% sucrose in the hardening medium

enhanced normal and active foliage development (1). Elevated CO_2 levels were shown to stimulate photoautotrophy (23), but this effect can be explained also as an antagonizing action to ethylene. The antagonizing effect to ethylene of CO_2 can stabilize the metabolic activity involved in lignification (1) and therefore may contribute to the prevention of hyperhydration, hypolignification, abnormal cell wall and aerenchyma formation, all events that may detemine abnormal morphogenesis in tissue cultured plants.

Acclimation of Micropropagated Plants. Substantial numbers of micropropagated plants do not survive transfer from *in vitro* conditions to greenhouse or field environments. The greenhouse and field have substantially lower relative humidities, higher light levels, and septic environments that are stressful to micropropagated plants compared to *in vitro* conditions. Most species grown *in vitro* require an acclimation process in order to insure that sufficient numbers of plants survive and grow vigorously when transferred to soil.

Plantlets or shoots that have grown *in vitro* have been continuously exposed to a unique microenvironment that has been selected to provide minimal stress and nearly optimal conditions for plant multiplication. Plantlets develop within culture vessels under low levels of light, aseptic conditions, on a medium containing ample sugar and nutrients to allow for heterotrophic growth, and in an atmosphere with high relative humidity. These all contribute to a phenotype that cannot survive the environmental conditions when directly placed in a greenhouse or field. Thus it is necessary to acclimatize plantlets gradually to ensure survival until they develop new leaves that are more adapted to the ambient conditions under which plants are normally grown.

The leaves that develop *in vitro* generally are lacking well developed epicuticular waxes, have raised stomata that may not close normally, have a poorly structured internal anatomy, and may not be photosynthetically efficient. These leaves never do become "normal" and it is imperative that new leaves that are more like those that develop on the greenhouse- or field-grown plants develop relatively quickly on the micropropagated plantlets.

Because leaves that form *in vitro* are poorly adapted to greenhouse conditions, it is usually necessary to provide conditions for the new plantlets that approximate the *in vitro* environment when they are first removed from culture. Plantlets are therefore acclimatized under high relative humidity and low light. However, it is important that the new leaves that form on the plantlets develop in conditions approaching those in which the plant will finally be grown. As new leaves grow on the plantlets it is best to reduce the relative humidity gradually and increase the light level to that of the environment under which the plants will grow.

Kozai (1988) has successfully micropropagated plants by creating conditions such that the plants grow photoautotrophically. This is accomplished by elevation CO_2 in the culture vessel, raising the light levels, and growing the plantlets on medium without sugar. These conditions may lead to an increased growth and survival rate when the plantlets are transferred to ex vitro conditions (31). Consequently, the conditions under which most laboratories micropropagate plants, i.e. high sucrose and nutrients, low light, no supplemental CO_2, and high relative humidity *in vitro*, may all contribute to problems encountered with weaning plants from the *in vitro* environment.

Off-type Plant Production. Tissue culture production of plants can result in production of off-type plants. Permanent differences, genetic in nature, may be due to tissue culture-induced stress, such as mitotic asynchrony, selection of

off-type cells by culture conditions, or by expression of previously existing variation, e.g. chimeras. This variation is mostly deleterious (45), but can be of great value to the breeder, except when variation is unwanted, e.g. after transformation, protoplast fusion, etc. For the propagator, testing clones for off-types, as done in several nurseries, is especially warranted when off-type plants have been obtained previously from a related cultivar or species or from seedlings obtained from the subject clone.

Temporary differences, which mimic physiologic or ontogenic variations, may be more useful. The rejuvenation of tissue culture-propagated plants is of value to plant propagators. The enhanced runnering of tissue culture-propagated strawberries can double the productivity of the nursery bed (54). Tissue culture-propagated lowbush blueberries produce rhizomes, unlike adult cuttings, and this response allows replanting of bogs with clonal plants. The yield reduction observed in other crops, e.g. sweet potato, is severe enough to preclude propagation by tissue culture unless plant cost is significantly lower (55). Plagiotropic growth is an unwanted consequence of tissue culture propagation in some conifers and in some propagation schema (56). A critical economic analysis of the benefits of the use of tissue culture versus the cost differential due to tissue culture propagation is obviously warranted. It may be possible to alter these equations by *in vitro* addition of plant growth regulators to "pre-program" the ex vitro behavior of tissue culture-produced propagules.

Sufficient testing of tissue culture-propagated material is absolutely essential. The results obtained from these tests must also be interpreted with a knowledge of genotypic and phenotypic capabilities, and the prevailing cultural systems in use.

CONCLUSION

Tissue culture applications are greatly dependent on improvements in basic technologies, which are developed by universities, companies and other research laboratories. However, the ability to raise internal or external funding for technology development seems to be increasingly dependent on previous successful applications. It appears in the future that much of the tissue culture basic research funding will come form increasingly applied programs.

There continues to be an need for improved micropropagation systems especially the solving of recalcitrant systems towards high shoot regeneration. What will be some of the scientific advances necessary to the economic application of tissue culture technology to a wide range of species? Some recent research suggests that manipulation of the stock plant environment, use of new methods of delivery of currently used and new plant growth regulating chemicals, and gaining a better understanding of the requirements for acclimation of tissue culture-derived plants show promise in improving tissue culture methods. As these and other emerging technologies become further investigated and adapted, it is likely that micropropagation will become a practical propagation method for an ever-increasing list of species. However, I believe that the long-term future of commercial micropropagation will revolve around somatic embryogenesis, particularly as a method to increase production efficiency and reduce costs. I wish to encourage applied research programs in this area. A commitment to tissue culture mechanization, materials handling and robotics research also should be made, particularly by industry.

Applications of tissue culture technology, particularly as it relates to plant improvement,

should be considered only as one stage in the overall scheme from product idea to market. Horticultural innovation is dependent on many disciplines working together, including plant breeding, genetics, cell biology and physiology, biochemistry and molecular biology, market research and marketing, and engineering.

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