Tissue Culture in the Nursery Industry©

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Tissue culture has limited application in the nursery industry for several reasons, but where it is applicable, both vegetative propagation efficiency and plant health status may be enhanced. Additionally, it may be used to rejuvenate aged material and also to generate novel but commercially valuable plants. Successful tissue culture of woody species is a difficult and slow process because of the many difficulties that have been encountered at the various stages of the process – culture initiation, regeneration, rooting, and acclimatisation. Additionally, plant physiological factors also impact on the process. For many woody subjects it is cheaper to propagate conventionally. Thus, protocols for many genera remain to be developed.

INTRODUCTION

Tissue culture technology can be applied to trees, shrubs, and herbaceous plants to increase multiplication efficiency in vegetative propagation, with the added potential of improving the health status of the plants. It involves the culture of explants (meristems, buds, nodes, leaf tissues, plant organs, cells, or callus) under aseptic conditions in which nutrients and plant growth regulators are supplied under specialised conditions. Tissue culture offers the potential to rapidly produce large numbers of selected plants and often induces physiological rejuvenation of the plant tissues leading to more vigorous plants. This may also be due to the direct or indirect elimination of systemic pathogens, which can affect production by conventional cuttings. The process of tissue culture can also be adapted to induce new mutations, which may be commercially valuable.

The technology for ornamental micropropagation has been primarily developed for herbaceous plants, especially interiorscape plants, pot plants, and flowers, which are required in large volumes. The technology has limited application in the nursery stock industry because of the many problems that have been encountered at the various stages of the process. However, protocols for some groups of trees and shrubs are already developed and have been applied commercially to important plant families (De Fossard et al., 1978; Anderson, 1984a; Maene and Debergh, 1985; Meier-Dinkel, 1992; and Morini et al., 1992). For others, either general or basic methods are available and many of the proposed pathways or protocols need to be adapted and remain to be developed or commercialised (Kerns and Meyer, 1986; Preece et al., 1988; Harris and Mantell, 1991; Morte et al., 1991; Geneve et al., 1997; and Nobre et al., 2000). However, subjects from most families have been experimented with (George, 1996). For ornamental grasses, the technology developed for species such as *Miscanthus* (Gawel et al., 1990) may be directly applicable and little adaptation may be required. However, success is often dependent on the responsiveness of specific plant organs and parts, coupled with induction systems that may or may not result in faithful replication of the genome.

Particular technical challenges in micropropagating woody plants include:

- Unresponsive taxon or genotype/clone or lack of suitable culture medium.
- Failure of explant growth.
- Inability to effectively surface disinfest plant material.
- Poor adaptability of explants to in vitro culture conditions.
- Latent bacterial contamination of established tissue cultures.
- Recalcitrant nature of adventitious root formation in many woody species.
- Slow growth rates in micropropagated plants and / or poor root development.
- Inactive plant physiological processes associated with the re-establishment of autotrophic conditions such as restricted water transport and stomata response leading to 'transplant shock'.
- Growing-on weaned nursery stock plants to a saleable stage using commercial nursery practices.

GENOTYPE AND CULTURE MEDIUM

It is generally accepted that tissue growth and multiplication in vitro is more influenced by genotype than by any other factor. For instance, organogenesis and rhizogenesis varies greatly between and within species and also from clone to clone. This phenomenon particularly manifests itself where morphogenesis from an intermediate callus phase occurs. Different callus textures and colour have been obtained from related plant cultivars.

There is also the effect of the environment, especially the growing medium. Plant growth media are formulated from a broad range of ingredients — macro- and micronutrients, amino acids, vitamins, growth regulators, a carbohydrate source, a gelling agent, and water. Occasionally, undefined substances such as banana, coconut, yeast, and potato extracts are added. Controlling the relative alkalinity and acidity of the culture medium is also paramount. Many plants may be cultured on the standard Murashige and Skoog, (1962) medium. It has provided the basis for many diverse formulations that have been prepared to improve the growth and morphogenesis of many different plant genera and species. Although all of these ingredients, either individually or collectively, influence culture establishment, growth and development, it is the inclusion of growth regulators that exerts the greatest influence on explant and propagule growth and development.

Ever since Skoog and Miller (1957) demonstrated that by varying the concentrations and balance of auxins and cytokinins in the culture medium, the pattern of organogenesis could be regulated and controlled. This has been a fundamental and guiding principle in tissue culture and is accepted as a general response in all plant species. However, the effects of growth regulators within the culture medium are not absolute and may simply modify endogenous levels, a phenomenon which appears to be heritable over many generations leading to the concept of habituation. The culture response can also be influenced by many other factors: the genotype; the ontogenetic age of the explant; the explant type (cells, tissues or organs); the location on the mother plant from which it was excised; the mother plant treatment prior to explant excision; explant polarity; and culture medium and environmental conditions. In herbaceous plants, many of these factors are not obvious because of

the inherent ease of micropropagating them. However, they are critical factors for woody species and must be overcome if commercially viable tissue culture systems are to be developed.

CONTAMINATION

Plants are invariably infested with contaminant organisms, especially bacteria, fungi, and invertebrate animal pests. Generally, where explants such as seeds, stem sections, or flowers are used, the sterilisation procedure is immediately effective. Similarly, delicate explants such as meristems, shoot tips, or immature ovules (which are enclosed in a tissue sheath) are usually contaminant free. However, explants from many woody plants are extremely difficult to disinfest, especially bud scales, sticky buds, or shoot tips with indumentum such as rhododendron. Similarly, plant material growing adjacent to the soil is also difficult to disinfest.

Recently, methods of double disinfestation, sequential in vitro generational manipulation, in vitro grafting, the use of antibiotics and a range of cultural growing methods for stock plants have been experimented with for diverse plant genera. But the new protocols developed may not necessarily be commercially feasible.

The phenomenon of latent disease infection/contamination in established tissue cultures is recognised as a major problem since it occurs spontaneously, despite the employment of a rigorous aseptic technique. Latent or endophytic infections do not immediately reveal their presence and in fact may not become evident until many sequential subcultures have taken place. For example, bacterial infection is often not immediately discernable and often requires time to adapt to in vitro conditions when subculture conditions become favourable for its development. Such infections are very destructive of cultures and can result in tissue necrosis, growth and rooting retardation, or even culture death (Long et al., 1988). Various methods have been used to minimize the potential for its occurrence, the simplest being meristem culture, where shoot tips up to 1.0 mm in length are generally contaminant free. Other methods include double disinfestation of the explants, the use of antibiotics, and photoautotrophic growing techniques. Opinions vary among researchers as to the efficacy of antiobiotics to control infections. It has been suggested that where antibiotics have been used, problems such as stem brittleness can occur in woody plants leading to plant collapse even after several year's growth in the field.

CULTURE MAINTENANCE

Many problems arise both in initiating and maintaining tissue cultures. These include explant discoloration, vigour decline, necrosis, and vitrification. Tissue discoloration is associated with complex mixtures of phenolic substances that are released or synthesized when tissues are wounded (Lerch, 1981). Several solutions to the problem have been suggested and range from reducing explant damage during disinfestation, etiolating mother plants, omitting copper and iron from the medium, reducing the electrical conductivity of the medium, the addition of antioxidants or a combination of these factors. Where discolouration occurs, frequent sequential transfers, coupled with tissue trimming, have been successfully practiced.

Some cultures exhibit growth decline and cease to grow and proliferate. According to Reeves et al. (1983) this can occur at any stage during culture (initiation or proliferation). On the contrary, where excessive proliferation occurs, (cytokinin habituation), the numerous small shoots produced often fail to root. Necrosis frequently

leads to the death of the whole culture. It frequently occurs in shoot tissues of woody plants, the most common reason being a lack of calcium absorption or translocation. The principal solution lies in making it more available to the explant, either through the use of liquid culture media or two-phase systems using liquid and solid formulations together.

Vitrification or glassiness of the tissues reduces culture success. It affects both herbaceous and woody plants alike. Low irradiance levels, high temperature, and relative humidity within the culture vessels, low plantlet transpiration rates, the use of liquid media versus solidified ones and the effects of carbohydrates have been associated with its occurrence. Other possible causes are the nutrient medium composition, its hydrogen ion concentration, and the lack of oxygen in the growing environment. These factors may contribute to the problem singularly or synergistically. However, it is accepted that where cultures require specific techniques for establishment and maintenance, the potential for vitrification to arise increases greatly.

ROOT FORMATION

The recalcitrant nature of adventitious root formation in many woody species is well known. A successful tissue culture system involves faithful replication of the propagule together with its efficient autotrophic establishment. Essentially, there are three routes to root initiation and growth — in vitro, "in vitro soil", and in vivo.

Many factors affect in vitro rooting. Investigations have centered on the culture medium, its growth regulator content, concentration and ratio, method of application, and the culture vessel environment together with tissue-dependent factors. In vitro rooting is the most commonly used method for rooting propagules despite its disadvantages. The main disadvantage is the fact that it is difficult to produce a fully effective root system capable of autotrophic carbon assimilation when the plants are transferred to a horticultural substrate. McClelland et al. (1990) reported that in vitro-produced roots often lack root hairs, stem vascular connections, and secondary cambium. Thus, root systems formed in vitro may not be sufficiently developed to support the plantlet growth, resulting in high mortality rates during the acclimatisation stage. Additionally, many roots die following transplanting, either naturally or because of physical damage during the procedure. A process known as in vitro hardening, accomplished through a range of techniques, can be employed to minimise transplant shock and increase plantlet survival when explanted. However, since additional plant manipulations are required, they are of limited commercial value.

Berney, (2002) reported a new concept known as "in vitro soil" rooting. This method seeks to initiate roots in a sterilised commercial propagation substrate prior to transfer to a nursery substrate. This system is intermediate between in vitro and in vivo rooting methods and facilitates oxygenation of the roots thus leading to a root system where secondary growth has taken place. The system may have broad application in re-establishing autotropism for woody plant species.

In vivo rooting uses commercial technology currently used in the nursery stock industry: humidity control using low-pressure and high-pressure fog; open and enclosed mist and polythene tents or covers; shade control using a range of netting materials or other proprietary products. Within such tents, or chambers, temperatures of 25 ^{o}C, light levels of 40 $\mu mol \cdot m^{2} \cdot s^{-1}$ and a relative humidity of 90% can be maintained. Basal heat and supplementary CO_2 may be provided. The optimum

greenhouse and substrate temperature together with the application or not of rooting hormones (in various forms and concentrations) is also influential.

It is generally accepted that in vivo rooting significantly reduces production costs since rooting and acclimatisation are effectively combined into a single operation. This system of rooting is widely practiced commercially.

WEANING

The establishment of plant autotropism frequently presents difficulties because tissue culture plantlets (microcuttings) suffer high mortality rates if they are transplanted directly from an in vitro environment to standard nursery propagation conditions. This arises because the leaves on tissue culture plantlets differ physiologically and anatomically from those raised under commercial nursery conditions. Sutter and Langhans (1982) reported that less wax was produced on in vitro produced leaves. It is this lack of wax that causes tissue-cultured plants to lose water rapidly through transpiration following transfer to an autotropic environment.

Sallanon et al. (1993) reported that stomatal number is reduced and that stomatal morphology, structure and function are irregular on tissue cultured leaves. For example, it is generally held that stomates on ex vitro plantlets are locked open and are unable to respond to normal stimuli thus resulting in rapid water loss post ex vitro planting. Similarly, photosynthetic competence also requires time to become effective following transfer from in vitro conditions.

Tissue culture plantlets often do not possess a functioning and competent root system (Smith et al., 1991). This limits acropetal water transport and results in plantlet death or transplant shock. Shoot survival then requires the new production of in vivo roots. Plantlet survival therefore, depends upon the ability of the micro cutting to withstand desiccation, assimilate carbon, and maintain photosynthesis.

Prior to transplanting, in vitro-produced plantlets should be of sufficient size and vitality and capable of acclimatising in vivo. Factors affecting this process include gel removal, physical and aeration characteristics of the substrate, and the rooting and establishment environment. These requirements are broadly similar to those commercially used for nursery propagation, but need to be refined and dedicated for the specific use. For example, a sealed greenhouse equipped with complete control over light, shade, temperature (basal and ambient), carbon dioxide supplementation and high humidity (preferably provided by a high pressure fogging system) is necessary. A well-aerated low bulk density substrate such as rockwool or perlite or other product combination is paramount. Anti-transpirant films may also have application.

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