significant reddening of the foliage. Growth of the unfertilised plants terminated after 15 weeks, in both cell sizes and this observation was consistent for plants exflasked in April, August, and November. Data from the February exflasking was not available when this report was presented, but early indications from appearance of the plants are that this trend will be continued, and growth will terminate in unfertilised plants before the end of the trial.

CONCLUSION

An understanding of the unique requirements of tissue culture exflasks is essential for successful establishment of plants in the greenhouse. In this work, a number of factors have been identified which affect survival and growth of blueberry plants. Quality of the plants that come from the laboratory has an influence on quality of the in-vivo plantlets (Kyte and Klein, 1983), but ultimately success of the exflasking process depends on skill of the grower. Good techniques for acclimatizing micropropagated blueberry plants are essential if the effort spent developing cultures to the exflasking stage are to be of benefit (Armstrong, 2001). With care and understanding, large numbers of healthy clonal blueberry plants can be produced economically through tissue culture.

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Acclimatizing Tissue Culture Plants: Reducing the Shock©

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INTRODUCTION

Many tissue culture milestones have been achieved over the past 50 years (Gamborg, 2002), with micropropagation established as a commercially viable form of vegetative propagation since the 1970s. Although many species are propagated using this technology there are still many more species that are either recalcitrant or cannot be cost-effectively propagated by tissue culture. Consequently, the commercial application of tissue culture propagation is restricted to mass propagating, high value, superior genotypes, and/or high health lines.

Plant tissue culture relies on growing microbe-free plant material in a sterile environment, in conjunction with defined media containing nutrients, growth promoters, and a carbohydrate source. Typically plant tissue culture is carried out on

a gelled medium within an enclosed clear or translucent culture vessel with limited ventilation, and placed under fluorescent lighting. To negate the need for plants to be photosynthetically active, or even photosynthetically competent under artificial lighting, sucrose is added to the medium as a carbohydrate source.

Major differences exist between the environments of plants growing in tissue culture and those in a greenhouse. These include differences in lighting, both quantity and quality; relative humidity; nutrients and other growth promoters; the gaseous composition; and the medium substrate. In addition, the rooting procedure differs markedly. In greenhouses a high-auxin quick dip is used for rooting cuttings. Excess auxin is flushed away in the free-draining, aerated potting medium. This contrasts to in vitro rooting where a low auxin concentration is available over several weeks in a poorly aerated, gelled medium. Therefore, it is not surprising that the transfer of plantlets, whether rooted or not, from the tissue culture environment to the greenhouse causes tissue stress and is often associated with slow growth and significant plant losses. This period of plant stress often coincides with a change in plant ownership following the sale of the plants by the tissue culture laboratory to the end, or intermediate, user. Consequently, the tissue culture conditions that the plantlet has been grown in and the conditions the plants are to be transplanted into are often poorly understood by the two parties. Differences between the two environments and their effect on plants have been recognised in numerous studies that aim to understand the factors involved in the transition and establishment of tissue culture plantlets into a standard greenhouse environment and improve the success rate. This paper reviews some of these factors.

WATER STRESS

The wilting of explants recently transferred from the in vitro to the ex vitro environment is an early symptom of poor plant re-establishment. Even though free water is available to these explants, incomplete leaf and stem development during the period in tissue culture contributes to the problems. Under standard tissue culture conditions, where the relative humidity is usually greater than 95%, in vitro leaves may not develop a waxy cuticle to the same extent as that found in ex vitro plants (Gilly et al., 1997). Nonacclimatised in vitro date-palm leaves have only 15% of the wax deposits found on greenhouse-grown seedlings (Zaid and Hughes, 1995). Ventilation of the culture vessel, using loosely fitting closures or vents, reduces the relative humidity in the vessel. A reduction in relative humidity leads to increases in plant transpiration with associated development of functional stomata for controlling plant water loss. This does not necessarily reduce total plant water content since *Malus* plantlets acclimatised by reducing the relative humidity before transplanting had a higher relative water content after transplanting, which contributed to both higher survival and improved net assimilation rates (Diaz-Perez et al., 1995b). Besides increasing the rates of in vitro transpiration, reducing the relative humidity can also increase nutrient uptake (Cassells and Walsh, 1994). Calcium and magnesium levels in *Delphinium* tissues are higher in plantlets grown in vented vessels than in plantlets grown in sealed vessels with high relative humidity (Santamaria et al., 2000). Stomatal performance was also improved along with subsequent growth and survival of plantlets.

ROOT DEVELOPMENT

In vitro root development usually enhances transplanting success because functioning roots can create a favourable plant water balance (Diaz-Perez et al., 1995a). The growth and survival of apple or rose microcuttings rooted ex vitro is lower than from in vitro-rooted microcuttings (De Klerk, 2000, 2002). Roots developed in vitro are believed to compensate for water loss caused by malfunctioning stomata. Improved performance and increases in dry weight of these in vitro-rooted plants may be due to extra nutrient uptake through the roots. After acclimatisation the water retention capacity of in vitro-formed apple leaves was lower than in vivo-formed leaves (De Klerk, 2002). In vitro rooting of *Malus* also facilitates the uptake of protective compounds such as putrescine, which result in greater fresh weight gains than in untreated tissue (De Klerk, 2002).

The presence of roots in culture does not always improve transplant success. Chestnut plantlets with roots developed in vitro had only a 50% survival rate compared to 100% survival of plants where roots developed out of culture (Goncalves et al., 1998). Inoculating plants at the time of transfer with mycorrhizal fungi can be beneficial for the survival and development of plantlets (Azcon-Aguilar et al., 1997), leading to functional stomata and increased photosynthetic rates compared to non-inoculated plantlets (Estrada-Luna, 2000). Strawberry plantlets inoculated in vitro and ex vitro out perform nonmycorrhizal plantlets in both fresh and dry weight gains (Cassells et al., 1996).

When the growth retardant, paclobutrazol, is added to the rooting medium shoots have fewer and smaller leaves. For citrus this reduction in plant growth makes them more wilt tolerant and increases the survival of plantlets following transfer to the greenhouse (Hazariki et al., 2002).

AUTOTROPHIC GROWTH

The gaseous environment within the culture vessel can influence in vitro tissue growth since plant tissue generates and absorbs gases, altering the gas composition. The level of gaseous exchange from a tissue culture vessel will depend on how well the vessel is sealed and whether it has vents. In addition, the type and amount of plant tissue in a culture vessel influences gas composition: ethylene may be generated, such as in response to wounding during tissue manipulations; carbon dioxide is a byproduct of respiration; and during photosynthesis oxygen is generated and carbon dioxide absorbed. Extensive studies at Chiba University have demonstrated the relationship between the extent of vessel closure and the ability of plant tissue to take up carbon dioxide for photosynthesis in the absence of sucrose in the medium (Kozai, 1991). They demonstrated that plantlets can grow in vitro in a sugar-free culture medium provided the environment is conducive to photosynthesis. In a closed vessel, plantlets are unable to achieve their photosynthetic capacity as the carbon dioxide concentrations are too low during most of the light period (Kozai et al., 1991). Increasing light levels and improving the availability of carbon dioxide, either through venting or artificial enrichment, allows plant tissue to be grown photoautotrophically in a vessel. Photosynthetic rates and growth of strawberry plantlets were greater under photoautotrophic conditions with carbon dioxide enrichment (Fujiwara et al., 1988). Photosynthesis is inhibited when sucrose levels increase from 3% to 6%, resulting in high levels of starch and sucrose in plantlets at the end of the in vitro period (Huylenbroeck and Debergh, 1996).

However, 2 weeks after transfer to the in vivo environment carbohydrate levels for plants cultured on either 3% or 6% sucrose were the same. Forced ventilation with carbon-dioxide-enriched air and high light resulted in sweet potato plantlets with a net photosynthetic rate five times higher than under low ventilation (Wilson et al., 2001). Under photoautotrophic conditions the survival and growth of sweet potato plantlets improved as they had functional stomata and waxier leaf cuticles compared to plants with low gaseous exchange in tissue culture vessels (Zobayed et al., 2000). Similarly with sugar-free media, in vitro carbon dioxide enrichment in association with relative humidity reduction promoted growth, including rooting, of transplants of red raspberry (Deng and Donnelly, 1993). Potato plantlets cultured on sucrose-free medium with high light and carbon dioxide levels were vigorous with good roots so that no particular rooting or acclimatisation processes were required following transfer to the greenhouse (Kozai et al., 1988).

Although leaves formed in vitro may be photosynthetically competent, these leaves are frequently replaced soon after transfer to the greenhouse by leaves with higher photosynthetic activity (Huylenbroeck et al., 1996). Leaves formed during the acclimatisation period may still have a lower photosynthetic capacity than leaves of greenhouse-grown plants (Carvalho et al., 2001). Increasing irradiance to around 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ immediately after transfer enhances photosynthetic activity (Carvalho et al., 2001). Chestnut plants acclimatised in vivo under elevated carbon dioxide levels (700 $\mu litre \cdot L^{-1}$) and irradiances (300 $\mu mol \cdot m^{-2} \cdot s^{-1}$) had a 100% increase in biomass compared to plantlets acclimatised at half these rates of carbon dioxide and irradiance (Carvalho and Amancio, 2002).

The optimum nutrient levels in a photoautotrophic system may be very different to those of a standard tissue culture system. Growth of carnation plantlets under high light levels was greater on a sugar-free medium using nutrient components widely used in hydroponic culture than either on a sugar-free medium with ½ Murashige and Skoog (MS) (Murashige and Skoog, 1962) or sugar containing hydroponic or ½ MS media (Kozai et al., 1988).

ALTERNATIVE PROPAGULES

Alternative propagules with little or no vegetative tissue present (e.g., bulbs or tubers) can be produced in culture with geophytes. As they sprout after transfer to the greenhouse, the new shoots are more tolerant to greenhouse conditions than similar shoots produced in culture. Bulblets of *Lilium* (Stimart and Ascher, 1978) and hyacinth (Pierik and Ruibing, 1973) form in vitro directly on bulb scales in culture. Continuous darkness increased both lily bulb size and number. We have produced *Zantedeschia* microtubers in vitro from tissue culture plantlets. With media adjustments the vegetative growth senesces and tubers form over a 3- to 6-month period. Besides the ease of acclimatisation, the compact and relatively robust form of microtubers makes them easy to handle and transport. They store well, allowing for greater flexibility in time of planting. In addition, they can be quickly scatter planted onto prepared greenhouse beds without the need for planting tubers upright. For rapid and even sprouting for in vitro bulbs and tubers, dormancy breaking needs to be synchronised. A period of cool storage is frequently used prior to planting out. Bulblets of Asiatic lily produced in culture and then cool stored in culture for 4 weeks transplanted with 100% success (Varshney et al., 2000).

SUMMARY

It is important to recognise and understand the differences between an in vitro and a greenhouse environment. By manipulating the in vitro environment, leaves that have greater tolerance to water stress and are photosynthetically competent can be developed as part of the acclimatisation process in preparing plantlets for transferring out of culture. Roots formed in culture can be beneficial for enhancing early growth following transfer from culture. The optimum growth rate of deflasked plantlets frequently does not occur until new leaves and roots develop in the greenhouse environment. However, as species differ greatly in their requirements there is no universal acclimatising protocol. However, for some crops alternative propagules (e.g., microtubers) are an option as they do not experience the acclimatisation problems observed in tissue culture plantlets.

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