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AERATION: A SIMPLE METHOD TO CONTROL VITRIFICATION AND IMPROVE IN VITRO CULTURE OF RARE AUSTRALIAN PLANTS

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SUMMARY

Aeration of tissue cultured rare Australian plants *Conostylis wonganensis* S.D. Hopper (Haemodoraceae); *Diplolaena andrewsii* Ostenf.; *Drummondita ericoides* Harvey (Rutaceae); *Eremophila resinosa* F. Muell. (Myoporaceae); *Eucalyptus 'graniticola'* (Myrtaceae); *Lechenaultia pulvinaris* C. Gardner (Goodeniaceae); and *Sowerbaea multicaulis* E. Pritzel (Liliaceae) has been found to reduce vitrification in sensitive species as well as significantly improving shoot quality and transfer to soil in most study species. A simple 7-mm hole with a double-layer insert of filter paper in the polypropylene screw lids of the culture vessel decreased shoot vitrification over a 4-wk culture period. The method has implications for facilitating the tissue culture of other rare Australian plants and reducing the occurrence of this developmental abnormality.

Key words: aeration; vitrification; rare Australian plants.

INTRODUCTION

Two-hundred and eighty of the 8000 known plant taxa found in Western Australia are considered rare and endangered (Hopper et al., 1990). Because not all species can be propagated by conventional seed and cutting methods, tissue culture has been developed for propagation of many of these species (Bunn et al., 1989; Bunn and Dixon, 1992).

A frequent problem affecting the success of in vitro propagation of rare Australian species is vitrification (Williams and Taji, 1991). Symptoms of vitrification include chlorophyll deficiency, cell hyperhydricity, hypolignification, reduced deposition of epicuticular waxes, and changes in enzymatic activity and protein synthesis brought about by alteration in normal plant metabolic processes (Ziv, 1991a,b). Vitrified propagules are glassy, with thick, translucent, and brittle leaves that show excessive basal growth and callus formation (Paques and Boxus, 1987; Ziv, 1991b). Vitrified leaves lack distinct palisade tissue and have malformed stomata and enlarged parenchyma cells with extensive intercellular spaces (Kevers et al., 1984). As a result, such plantlets desiccate rapidly and die when transferred to soil. Vitrification can be controlled by reducing cytokinin (or balancing the auxin:cytokinin ratio) or sugar and nutrient levels in the medium, increasing agar concentration, and changing the concentration of other components such as chloride, cobalt, and calcium (Leshem, 1983; Leshem and Sachs, 1985; and *see* Ziv, 1991b for further details and references). Alteration of the culture environment e.g., cooling, ventilation, and aeration systems, can also reduce vitrification (Debergh and Vandershaeghe, 1990).

Aeration of cultures reduces the accumulation of ergastic gases that can lead to vitrification (Jackson et al., 1991). Dillen and Buysens (1989) found a simple and practical in vitro aeration technique (consisting of perforations in the lids of culture vessels) that resulted in reduced vitrification in *Gypsophila paniculata.*

Aeration therefore has the potential of being a simple and efficient method for improving the in vitro culture of a large number of plant species, especially those with persistent vitrification problems. The aim of this study was to assess the benefits of aeration in minimizing vitrification and improving plant quality in tissue culture of rare and endangered Western Australian plant species.

MATERIALS AND METHODS

Plant Material

Plant species used in these experiments were rare and endangered Western Australian flora. Cultures of *Conostylis wonganensis* S.D. Hopper and *Drummondita ericoides* Harvey were originated from apical shoot cultures derived from wild collected material. These two species had been in culture for 2 and 6 yr, respectively. *Sowerbaea multicaulis* E. Pritzel cultures originated from embryos dissected from wild collected seeds, and experiments were conducted on plants 4 wk after commencement of growth. *Diplolaena andrewsii* Ostenf., *Eremophila resinosa* F. Muell., *Eucalyptus 'graniticola'* (manuscript name), and *Lechenaultia pulvinaris* C. Gardner were derived from apical shoot cultures from wild collected material and, except for the first species, had all been in culture for less than 6 mo. All species (except *S. multicaulis*) were last subcultured 4 wk before experimentation.

Media and Culture Conditions

Basal medium for all experiments was modified half-strength Murashige and Skoog (1962) with supplementary vitamins (thiamine-HCl 3.0 μM; pyridoxine-HCl 2.5 μM; niacin 4.0 μM), 20 $g \cdot liter^{-1}$ sucrose, 0.5 μM 6-benzyl adenine, pH 6.0, 10 $g \cdot liter^{-1}$ agar (plant cell culture tested, Sigma, St. Louis, MO, no. A-9799) and autoclaved at 121° C and 1.02 $kg \cdot cm^{-2}$ for 20 min. The cultures were maintained in glass jars (diameter

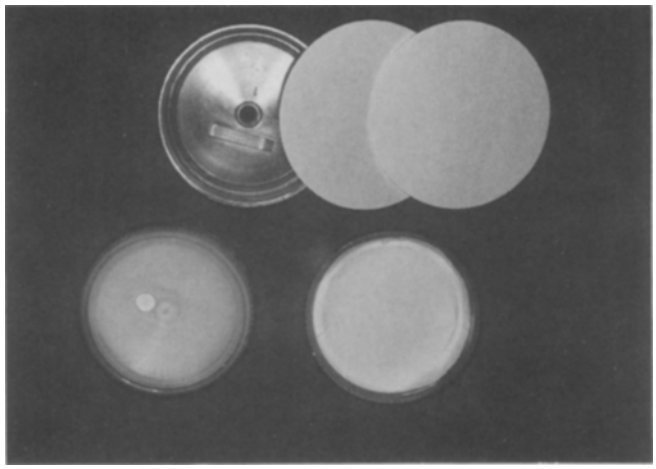

FIG. 1. Aeration lids before and after assembling, showing the perforation and filter paper inserts.

of opening 55 mm, medium surface 1105.5 mm^2) with polypropylene screw-on lids containing 25 ml of medium. Each jar contained five plantlets. The cultures were maintained under cool-white fluorescent tubes (16 h light at 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$, 8 h dark) at 22° to 25° C.

Experimental

Vitrification. Non-vitrified plantlets were selected and subcultured in equal numbers into jars with either entire or perforated lids. Perforated lids (Fig. 1) had a single, central 7-mm circular hole and two circular filter paper inserts (7.0 cm Whatman qualitative no. 1). Jars with lids having 2, 3, 4, or 5 holes had been experimented with in a preliminary trial but were discarded because of excessive medium desiccation and shoot decline during the normal subculture period. The perforated lid could be screwed on and off without affecting the position of the filter papers. Strips of transparent food wrap were placed around the edge of both types of lids. After 1 mo., the number of vitrified plantlets and the multiplication rate in both types of jars were scored.

Shoot quality. D. ericoides (a dicotyledon) was considered vitrified when more than 25% of the leaf and shoot surface area showed the symptoms described in Table 1, whereas for the two monocots (*S. multicaulis* and *C. wonganensis*) the vitrification symptoms were always found over the entire surface of all leaves. Qualitative assessment of the use of aeration for tissue culture was also undertaken on the other four plant species (*D. andrewsii, E. resinosa, E. 'graniticola'*, and *L. pulvinaris*) that exhibited varying degrees of vitrification.

Transfer to soil. After 4 wk of in vitro culture nonaerated and aerated shoots of *D. ericoides, S. multicaulis,* and *D. andrewsii* were transferred to pasteurized 1:1 peat/perlite mix, inside a fogged greenhouse. Survival of the plantlets was scored after 4 wk in the greenhouse.

Medium dehydration. To determine the rate of water loss from the aerated culture vessels, the weight of aerated and nonaerated jars, containing only culture medium and no plants, was scored twice a week for 4 wk.

Histology. One-micron thick transverse sections were cut from vitrified and normal *C. wonganensis* leaves and compared microscopically after embedding in glycol-methacrylate resin and staining with toluidine blue (O'Brien and McCully, 1981).

RESULTS AND DISCUSSION

In vitrified leaves of *C. wonganensis,* normal stomata were absent, the epidermal layer was loose and disrupted, and palisade cells were reduced to a single layer of loosely connected cells with large intercellular spaces between shriveled and distorted mesophyll cells (Fig. 2 *a*). In comparison, non-vitrified leaves of *C. wonganensis* had a well-defined epidermis with numerous stomata, two layers of compact palisade cells, and turgid mesophyll cells (Fig. 2 *b*). The microstructural differences between vitrified and non-vitrified leaf sections in *C. wonganensis* were similar to those observed in other Australian species (Williams and Taji, 1991).

When cultured in aerated jars, both *D. ericoides* and *S. multicaulis* produced significantly (X^2 test, $P < 0.005$) less vitrified plantlets than when cultured in jars with normal lids (Fig. 3). Shoot multiplication was similar in the two treatments, and aeration produced 2.5 ± 0.9 plantlets in *D. ericoides* (similar to the 2.1 ± 1.2 plantlets produced by the control treatment) and 2.1 ± 0.8 plantlets in *S. multicaulis* (similar to the 2.2 ± 0.8 plantlets produced in the control treatment). There was no significant difference (X^2 test, $P < 0.005$) in the number of vitrified *C. wonganensis* plantlets produced in aerated vs. nonaerated jars. However, aeration significantly reduced the number of vitrified explants in the other two study species (Fig. 3). Thus the aeration system successfully minimized vitrification in species normally showing high vitrification rates without adversely affecting growth or multiplication. It has been shown that ventilation of cultures reduced the risk of waterlogging and ethylene accumulation, which causes enzymatic inhibition and abnormal development of the plantlets (Kevers et al., 1984). It is possible that the small amount of vitrification exhibited by each species in the aerated trials was influenced by components of the medium, including agar type, that were not related to the gaseous phase. Also, some starting shoot material used in the experiment, could have been slightly vitrified and incapable of a return to normal growth during the trial (a problem common in other Australian species such as *Blancoa canescens* Lindley, *Conostylis*

TABLE 1

APPEARANCE OF VITRIFIED AND NON-VITRIFIED PLANTLETS OF THE THREE STUDY SPECIES USED IN THE VITRIFICATION EXPERIMENT

Species	Non-vitrified	Vitrified
D. ericoides	Slender shoots with 10- to 12-mm long pale green leaves	Shoots short, leaves thick, dark green, glassy and succulent, 8- to 10-mm long
S. multicaulis	Leaves thin, pale green, separate, 20- to 30-mm long	Larger number of shoots. Leaves dark green, wet, translucent and adhering
C. wonganensis	Plants 40- to 45-mm long, leaves erect, light green, hirsute	Plants contorted 25- to 30-mm long, leaves wet, glassy, glabrous, short and dark green

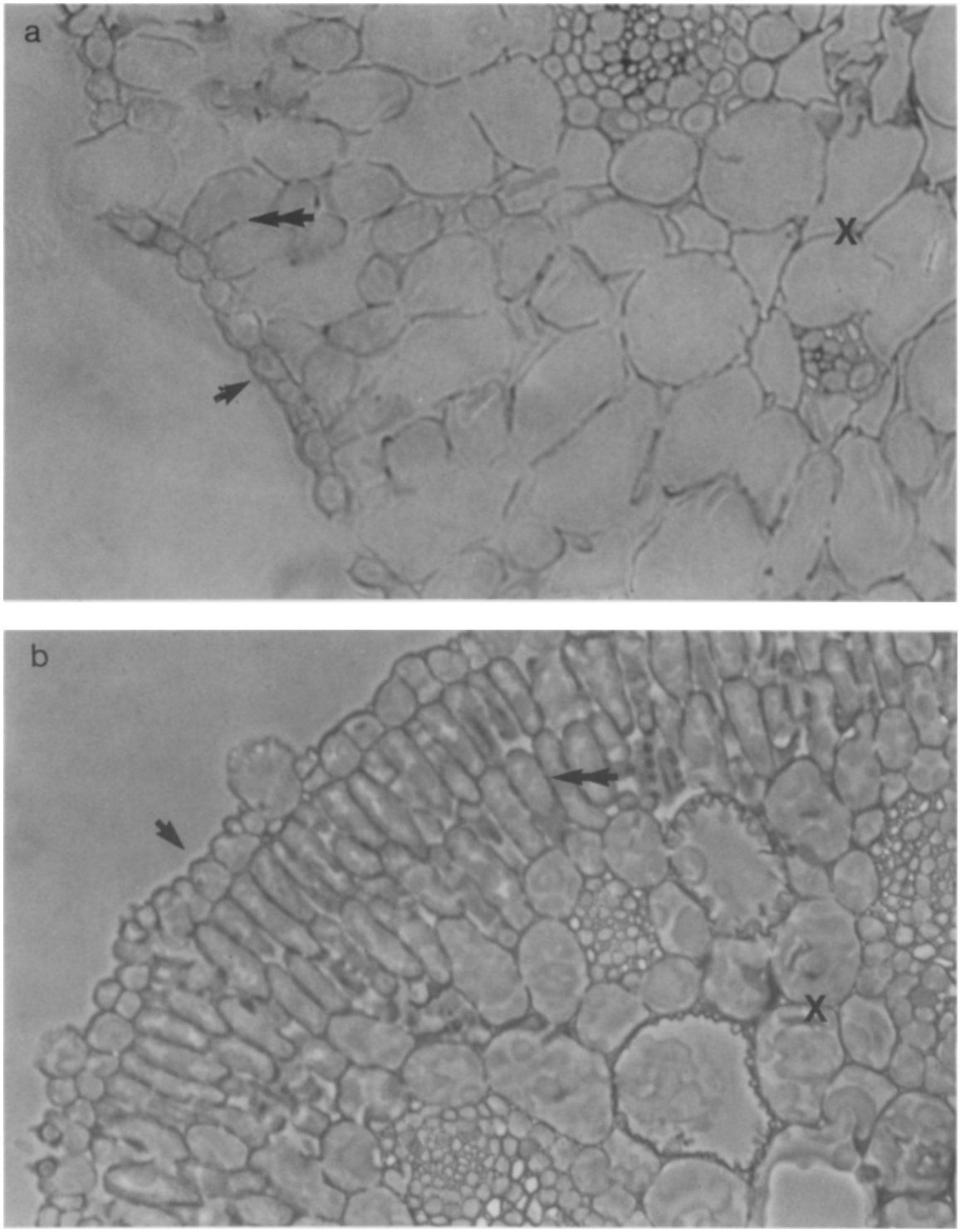

FIG. 2. Histological detail (1 μm GMA section, stained with toluidine blue) of vitrified and non-vitrified leaf tissue of *C. wonganensis*. *a*, Vitrified tissue shows breakages in the epidermal layer (→), a poorly developed palisade layer (→→) and loosely connected mesophyll cells (×). *b*, Non-vitrified tissue shows a well-defined epidermis with normally structured stomata, two layers of compact palisade cells and turgid mesophyll cells.

TABLE 2

DESCRIPTION OF SHOOT QUALITY IN IN VITRO PLANTLETS OF SELECTED RARE AND ENDANGERED SPECIES CULTURED UNDER STANDARD CONDITIONS WITH AND WITHOUT AERATION

Species	Aerated	Nonaerated
D. andrewsii	Shoots 55- to 65-mm long with dark, broad, hairy leaves	Shoots 25- to 30-mm long with dark green, smaller leaves
E. resinosa	Shoots 35- to 55-mm long. Leaves pale green, healthy and evenly spaced. Slightly greater shoot multiplication than in non-aerated cultures	Shoots approx. 25-mm long, leaves dark green, mostly basal; slight vitrification
E. 'graniticola'	Shoots approx. 15-mm long; many leaves and some basal growth	Shoots 4- to 5-mm long with fewer leaves and slight vitrification (especially in smaller shoots)
L. pulvinaris	Shoots approx. 15-mm long; plantlets with dry, compact, glaucous leaves	Shoots 30- to 35-mm long; pale green, etiolated and wet

misera Endl. (Haemodoraceae), *Pimelia physodes* Hook. (Thymelaeaceae) (E. Bunn personal communication). This was especially true in plants showing strong basal shooting, such as *C. wonganensis*, where vitrification was uncontrollable once the symptoms were evident. Caulescent plants with strong apical growth (e.g., shrub species) however, seemed to be capable of outgrowing the vitrifying process when placed in ameliorating conditions.

Assessment of shoot quality in aerated cultures of *D. andrewsii*, *E. resinosa*, *E. 'graniticola'*, and *L. pulvinaris* was encouraging and showed that plantlets were larger and healthier as a result of aeration, and vitrification only occurred in the control jars (as shown in Table 2).

After 4 wk, the weight loss of the media in the treatments during the experimental period was low (2.39% in the aeration trial and 7.2×10^{-4}% in the control) (Fig. 5). Using the formula of Dillen and Buysens (1989), the agar concentration increase due to evaporation is 2.45% in the aerated jars. This implied that, at normal levels, the type of agar used in this study was not the vitrifying agent and that gaseous exchange may have been the main factor influencing the incidence of vitrification.

In species where aeration helped to control vitrification there was a significant increase in shoot survival after transfer to soil (Fig. 4). However, despite producing a 100% survival ex vitro, the aeration treatment did not significantly improve the results obtained for the control using *D. andrewsii*, one of the species not showing definite vitrification under normal conditions. Hence, in general, the use of perforated lids seems to improve the hardening off process because aerated plantlets are likely to be acclimated to lower humidity and increased gas exchange.

The aeration system described here is a simple system that is contamination-free, controls vitrification, and improves in vitro culture and transfer to soil of shoots for most of the study species. If necessary this technique can be further developed for particular species by increasing or reducing the amount of gas exchange. This

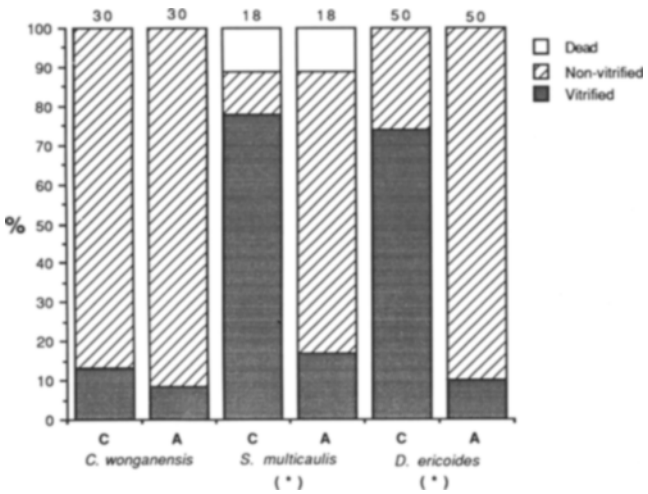

FIG. 3. Proportion of vitrified, non-vitrified, and dead shoots in control (*C*) and aerated (*A*) jars for the three study species. (*Asterisk*) shows significant difference (X^2 test, $P < 0.005$) in number of healthy shoots found in aerated and control treatments. Number of plantlets used in each trial is indicated at the top of each column.

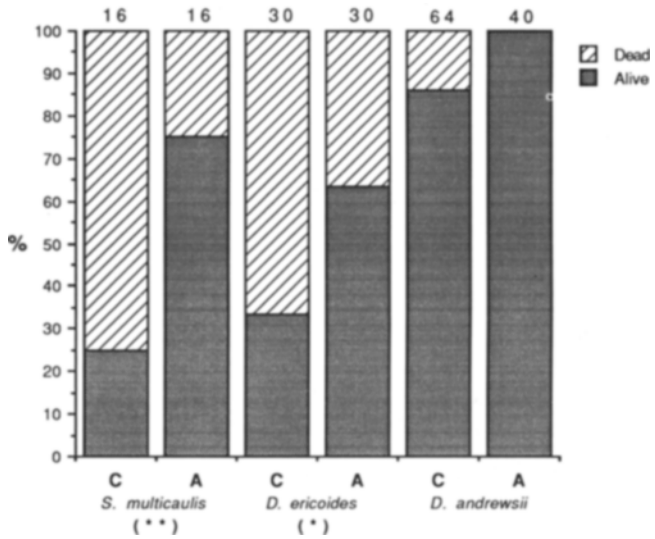

FIG. 4. Proportion of dead and living shoots, 4 wk after transfer to soil of control (*C*) and aerated (*A*) jars for three species. *Double asterisk* and *asterisk* indicate statistical difference (X^2 test, $P < 0.05$ and $P < 0.1$, respectively) in number of surviving shoots between control and aerated treatments. Number of plantlets used in each trial is indicated at the top of each column.

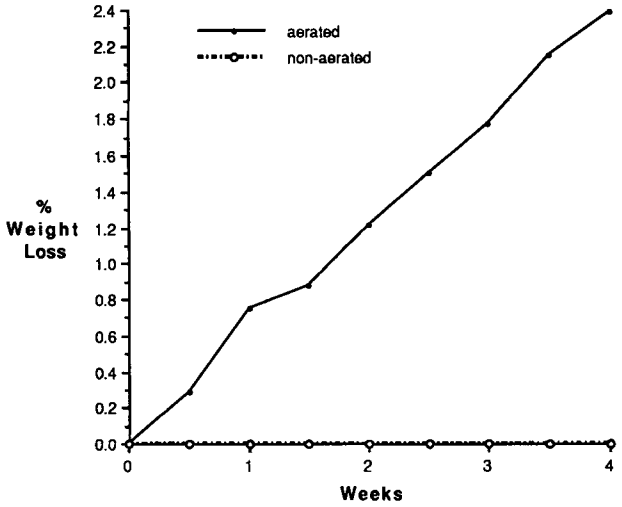

FIG. 5. Weight loss of standard culture medium in aerated and nonaerated jars, over a 4-wk period of incubation under standard culture conditions (*see* Materials and Methods).

could be done through adjustments in the number of holes or through the alternation of periods with and without aeration.

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REFERENCES

Bunn, E.; Dixon, K. W.; Langley, M. A. In vitro propagation of *Leucopogon obtectus* Benth. (Epacridaceae). Plant Cell Tissue Organ Cult. 19:77–84; 1989.

Bunn, E.; Dixon, K. W. In vitro propagation of the rare and endangered *Grevillea scapigera* A. S. George (Proteaceae). HortScience 27(3):261–262; 1992.

Debergh, P.; Vanderschaeghe, A. Mass propagation of in vitro plantlets. Chronica Hortic. 30:1–2; 1990.

Dillen, W.; Buysens, S. A simple technique to overcome vitrification in *Gypsophila paniculata* L. Plant Cell Tissue Organ Cult. 19:181–188; 1989.

Hopper, S.; Van Leeuwen, S.; Brown, A., et al. Western Australia's endangered flora. Department of Conservation and Land Management. W.A. Wildlife Research Centre, P.O. Box 51, Wanneroo, W.A., 6065; 1990.

Jackson, M. B.; Abbott, A. J.; Belcher, A. R., et al. Ventilation in plant tissue cultures and effects of poor aeration on ethylene and carbon dioxide accumulation, oxygen depletion and explant development. Ann. Bot. 67:229–237; 1991.

Kevers, C.; Coumans, M.; Coumans-Giles, M.-F., et al. Physiological and biochemical events leading to vitrification of plants cultured *in vitro*. Physiol. Plant. 61:69–74; 1984.

Leshem, B. Growth of carnation meristems *in vitro*: anatomical structure of abnormal plantlets and the effect of agar concentration in the medium on their formation. Ann. Bot. 52:413–415; 1983.

Leshem, B.; Sachs, T. 'Vitrified' *Dianthus*—Teratomata *in vitro* due to growth factor imbalance. Ann. Bot. 56:613–617; 1985.

Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 15:473–479; 1962.

O'Brien, T. P.; McCully, M. E. The study of plant structure: principles and selected methods. Melbourne: Termarcarphi; 1981.

Paques, M.; Boxus, Ph. "Vitrification": review of literature. Acta Hortic. 212:155–166; 1987.

Williams, R. R.; Taji, A. M. Effect of temperature, gel concentration and cytokinins on vitrification of *Olearia microdisca* J. M. Black in vitro shoot cultures. Plant Cell Tissue Organ Cult. 26:1–6; 1991.

Ziv, M. Quality of micropropagated plants—vitrification. In Vitro Cell. Dev. Biol. 27:64–69; 1991a.

Ziv, M. Vitrification: morphological and physiological disorders of *in vitro* plants. In: Debergh, P. C.; Zimmerman, R. H., ed. Micropropagation. The Netherlands: Kluwer Academic Publishers; 1991b:45–69.