CROP BREEDING AND APPLIED BIOTECHNOLOGY

ARTICLE

Influence of ethylene glycol on *Eucalyptus grandis* cryopreservation using the V cryo-plate technique

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Abstract: *The conservation of plant genetic resources is fundamental for the development of agriculture. The development of efficient cryopreservation protocols has become an effective tool to preserve cells, tissues and organs of different plant species. We sought here to develop an efficient method of cryopreservation of Eucalyptus grandis employing the V cryo-plate technique. This experiment examined the exposure of shoot tips to three different cryoprotectants (PVS2, PVS3, and PVS4). The cryoprotectants PVS2 and PVS4 proved to be more efficient among the cryoprotectants tested, as 44.4% of the shoot tips immersed in those solutions survived, and 31.1% generated new shoots. Ethylene glycol was found to be a key compound for the successful cryopreservation of shoot tips, in light of its low toxicity as compared to other cryoprotective compounds. Thus, the methodology developed here represents an effective method for the conservation of E. grandis germplasm through cryopreservation and the use of the V cryo-plate technique.*

Keywords: *Plant vitrification solution, genetic conservation, germplasm.*

INTRODUCTION

The genus Eucalyptus comprises more than 700 species, subspecies, varieties, and natural hybrids, and many of them have been introduced throughout the world, mainly in tropical and subtropical regions (Grattapaglia and Kirst 2008, Gonçalves et al. 2013). The large-scale planting of Eucalyptus taxa reflects their rapid growth, high productivity, and the multiple uses of their woods, and has, in turn, generated large investments by companies that use wood products in industrial processes (Grattapaglia and Kirst 2008).

Due to the great commercial importance of eucalyptus products, companies working on genetic enhancement of its species have also invested in methods for conserving genetic material and preserving selected genotypes (Padayachee et al. 2009). The long-term preservation of superior hybrids usually involves clonal propagation, but forest species maintained in clonal gardens or field plantations are often exposed to both biotic (pests and diseases) and abiotic (drought, storms, floods, high temperatures) stresses (Trueman et al. 2018). Those potential problems put *ex situ* conservation programs at relatively high risk in terms of the long-term species preservation – highlighting the importance of alternative technologies such as *in vitro* conservation to guarantee the

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maintenance of genetic material. Cryopreservation, therefore, presents itself as a technique potentially capable of minimizing damage caused by prolonged exposure to environmental vagaries (Rao 2004).

Cryopreservation involves storing plant material at ultra-low temperatures, such as in liquid nitrogen (-196 °C), a condition in which metabolic activities cease and no genetic alterations occur in the cryopreserved material (Engelmann 2004). That technique can complement other existing preservation methods and serve as a backup for broad genetic-based breeding programs, for the maintenance of superior clones during field trials, and the maintenance of endangered species (Padayachee et al. 2009, Carneros et al. 2017, Gross et al. 2017, Li et al. 2018, Bettoni et al. 2019). Additionally, cryopreservation requires only minimum space for genotype maintenance, is a relatively low-cost technique, has a low incidence of contamination, and limits somaclonal variation (Engelmann 1992, Pukacki and Juszczyk 2015, Ahn and Choi 2017).

Despite the many advantages of cryopreservation, viable protocols that can guarantee good rates of regeneration of the material after cryopreservation are not yet available for most species, thus limiting its application for long-term germplasm conservation (Funnekotter et al. 2017). Several factors can influence plant recovery after immersion in liquid nitrogen, including the age of the mother plant that provided the explant, resistance of tissues during dissection, the type of cryoprotectant solution employed, the time of exposure to it, and the specific cryopreservation techniques to be employed (Sakai and Engelmann 2007, Uchendu and Reed 2008, Melo et al. 2011, Engelmann 2014).

Plant explants can be cryopreserved using encapsulation-dehydration or vitrification techniques. The high applicability of the vitrification technique in several crops, as well as its rapid implementation, have led to numerous variations of this method, such as droplet-vitrification (Panis et al. 2005), encapsulation-vitrification (Fabre and Dereuddre 1990), vacuum infiltration vitrification (Nadarajan and Pritchard 2014), and the use of cryo-plates (Yamamoto et al. 2011). The use of aluminum cryogenic plates facilitates the handling of shoop tips, avoiding minimal injuries and optimizing the explant cooling and heating process, which will result in high survival rates and shoot tips development. This is because the shoot tips are fixed to the plate, facilitating their manipulation in the cryopreservation process (Gupta 2014). The cryo-plate method stands out as being simple (which facilitates the large-scale storage of cryopreserved explants) and for its regeneration percentages that reach 90% for some species (Yamamoto et al. 2011, Yamamoto et al. 2012, Engelmann 2014, Pettinelli et al. 2017). There are still no reports, however, of the cryopreservation of any Eucalyptus taxa using the cryo-plate technique.

Plant Vitrification Solutions (PVS) represent the most commonly used cryoprotective solutions. They serve the function of removing and replacing the water inside the cells before the freezing process, while avoiding excessive dehydration – although all PVS have different modes of action that reflect their individual chemical compositions (Gross et al. 2017, Volk and Caspersen 2017). One of the most commonly used components in PVS solutions is ethylene glycol, as it easily permeates cell membranes and has low toxicity (Turner et al. 2001, Volk et al. 2006).

We therefore sought to develop an efficient method for the cryopreservation of *Eucalyptus grandis* shoot tips using the cryo-plate technique by testing shoot tips of different origins, evaluating different cryoprotectant exposure times, and determining the most efficient cryoprotectant solution.

MATERIAL AND METHODS

Explant origins

We used shoot tips originating from 30-day-old seedlings that had been germinated *in vitro*. To obtain the shoot tips, *E. grandis* seeds were disinfected in 70% ethanol for 30 seconds, immersed in a 2.5% sodium hypochlorite solution with two added drops of commercial detergent, and then shaken for 20 minutes. The seeds were then triple rinsed with sterile distilled water in a laminar flow chamber and the excess water was removed using sterile filter paper. The seeds were subsequently inoculated for germination onto 40 mL of semisolid MS medium (Murashige and Skoog 1962), supplemented with 30 g L^{-1} of sucrose and 4.0 g L^{-1} of agar (adjusted to pH 5.8). The seeds were kept illuminated for 20 days and were allowed to germinate, after which their shoot tips were removed.

The shoot tips (1-2 mm) were excised while being viewed under a stereo microscope (in a laminar flow chamber) and

were maintained in MS medium, plus 30 g L^{-1} of sucrose and 4.0 g L^{-1} of agar (pH 5.8) until initiating the cryopreservation process.

Cryopreservation using the cryo-plate technique and different cryoprotectants

The shoot tips were subjected to a dehydration pre-treatment to remove some of the cellular water before the freezing step. To that end, the shoot tip was held for 24 hours in each of two semisolid MS media with high concentrations of sucrose (0.25 M and 0.5 M), in the dark at 26 ± 2 °C.

To encapsulate the shoot tip, a 3% alginate solution containing 30 g L^{-1} sucrose in calcium-free MS medium was pipetted into each cryo-plate well. The aluminum cryo-plates used were 7 mm × 37 mm × 0.5 mm, with 10 wells each (well diameters 1.5 mm, depth 0.75 mm). One shoot tip was then placed in each cryo-plate well and covered with 3.0 µL of a 100 mM calcium chloride solution. The shoot tips became encapsulated within 30 minutes and adhered to the cryo-plate.

The cryo-plates containing the shoot tips were then immersed in a loading solution (2 M of glycerol and 0.4 M of sucrose in MS medium) for 15 minutes at room temperature, and subsequently immersed in the cryoprotectant. Three different cryoprotectants, PVS2, PVS3, and PVS4, were used (Table 1), and incubated at 0 °C for 60 minutes. The cryo-plates were then transferred to 2.0 mL cryo-tubes containing liquid nitrogen and those were immersed in liquid nitrogen for one hour.

After one hour, the cryo-plates were removed from the liquid nitrogen and immediately immersed in a recovery solution (MS medium containing 1.2 M of sucrose), where they remained for 15 minutes at room temperature. Then, to start the shoot tip rehydration process, the cryo-plates were placed in liquid WPM medium (supplemented with 50 g L^{-1} of sucrose, 100 mg L^{-1} of ascorbic acid, 0.5 mg L^{-1} of BA, and 0.02 mg L^{-1} of NAA [at pH 5.8]) to remove possible residues from the previous solution. After that washing, the shoot tips were removed from the cryo-plates, inoculated into Petri dishes containing WPM semi-solid medium (Lloyd and McCown 1981) with 30 g L^{-1} of sucrose, and held under low light for five days in a growth room (photoperiod of 16 hours of light, at 26 ± 2 °C). They were then transferred to direct light, where they remained during the regeneration process.

Table 1. Composition of the cryoprotectants used in the treatments

Cryoprotectant	Composition*	Reference
PVS2	MS Medium, 0.4 M Sucrose, 30% Glycerol, 15% DMSO (dimethyl sulfoxide), 15% Ethylene glycol	Sakai et al. (1990)
PVS3	MS Medium, 0.5 M Sucrose, 50% Glycerol	Nishizawa et al. (1993)
PVS4	MS Medium, 0.6 M Sucrose, 35% Glycerol, 20% Ethylene Glycol	Sakai (2000)

* All cryoprotectants had their pHs adjusted to 5.8.

Experimental evaluations

Survival percentages were evaluated 15 days after cryopreservation; shoot tip regeneration was evaluated after 30 days. The culture medium was renewed at that time, using the same formulation.

After 60 days, the regenerated shoots were transferred to glass jars containing the same WPM medium used in the plates, and the numbers of shoots per shoot tip were counted. After 90 days, the shoots were then transferred to other jars containing semisolid WPM medium, supplemented with 0.01 mg L^{-1} of BA, 0.1 mg L^{-1} of NAA, 0.1 mg L^{-1} of IBA, 20 g L^{-1} of sucrose, and 4 g L^{-1} of agar (at pH 5.8), for the elongation of the aerial part and rooting.

Statistical analyses

A completely randomized design (CRD) was used in the experiment, which tested different cryoprotectants, with each repetition using 24 shoot tips, totaling 72 shoot tips per treatment. The data from the experiments were subjected to analysis of variance, and means were compared using Tukey's test at a 5% level of significance, employing SISVAR software (Ferreira 2008) for the statistical tests.

RESULTS AND DISCUSSION

Cryopreservation is currently the only safe and economical means of long-term conservation of genotypes (Dixit et al. 2005). However, several factors can interfere in that process and in the recovery of material after freezing.

In order to improve the survival and regeneration rates of the shoot tip, and establish cryopreservation as a viable alternative for the preservation of *E. grandis*, three cryoprotectants (PVS2, PVS3, and PVS4) were tested in this experiment. Shoot tip from 30-day-old seedlings were used as explants, and the exposure time to the cryoprotectants was 60 minutes. Our results indicated that the different PVS solutions influenced the survival and regeneration percentages of the shoot tips, as well as subsequent shoot formation (Table 2). The cryopreservation steps and shoot tip regeneration data are summarized in Figure 1.

The cryoprotectants tested that had the highest survival percentages were PVS2 and PVS4, with 45.5% and 43.33% survival, respectively. Much lower shoot tip protection efficiency was obtained with PVS3, as none of the shoot tips exposed to that cryoprotectant survived after immersion in liquid nitrogen, although many of the shoot tips exposed to PVS3 for 60 minutes were able to survive (62.22%) and regenerate (53.33%), but not those that had been immersed in liquid nitrogen (LN^-).

In terms of the numbers of shoots produced (except for the shoot tips exposed to PVS3, which generated no shoots), the shoot tips exposed to all of the treatments regenerated multiple shoots, even those that had been immersed in liquid nitrogen. After regenerating shoots, the cryopreserved explants held in an environment that promoted elongation developed aerial parts and were able to subsequently root *in vitro* (Figure 1) – indicating that the cryopreservation method allows the regeneration of *E. grandis* mini-cuttings.

The cryoprotectant PVS3 proved to be the least efficient in protecting shoot tips during cryopreservation. PVS3 contains a high concentration of glycerol as compared to PVS2 or PVS4 (Table 1), but that component was not sufficient to guarantee apex survival. Glycerol has been shown to be effective in protecting some explants (Turner et al. 2001, Ellis et al. 2006, Volk et al. 2006), although it causes plasmolysis during cryopreservation in others (Volk and Walters 2006, Volk and Caspersen 2017), an effect due to its high molecular weight and significant viscosity (which makes it difficult to penetrate into the cells) (Kim et al. 2009).

The success of the cryopreservation of plant materials will depend on the combination of several compounds that can prevent further explant dehydration and facilitate vitrification during direct immersion in liquid nitrogen (Brison et al. 1995, Bekheet et al. 2020). It has been shown that the addition of DMSO and ethylene glycol to cryoprotectant solutions can increase the stability of the vitreous state of the water in the cell membrane, thus reducing intracellular ice formation (Turner et al. 2001, Kreck et al. 2011, Malajczuk et al. 2013). Both DMSO and ethylene glycol are penetrating cryoprotectants that show more rapid permeability through cell walls and the protoplast than compounds such as sucrose and glycerol (Tao and Li 1986, Volk et al. 2006). Additionally, ethylene glycol has low toxicity and, when used in combination with high concentrations of sucrose, improves the formation of the vitreous state in the cells and so favors greater explant survival (Turner et al. 2001, Oliveira et al. 2003). DMSO is present in PVS2, and ethylene glycol is present in both PVS2 and PVS4 at similar concentrations, indicating that the latter was essential for the success of *E. grandis*

Table 2. Cryopreservation of *E. grandis* seedling shoot tips using different cryoprotectants (PVS2, PVS3, and PVS4)

Treatment		% Survival	% Regeneration	**N. Shoots**
Control		81.11 a	72.22 a	45 a
PVS2	LN^-	61.11 a	47.77 ab	34 a
	LN^+	45.55 b	28.88 b	22 a
PVS3	LN^-	62.22 a	53.33 ab	32 a
	LN^+	0.00 c	0.00 c	0 b
PVS4	LN^-	52.22 ab	42.22 b	18 a
	LN^+	43.33 b	33.33 b	10 a

* Means followed by the same letters do not differ statistically by Tukey's test ($p<0.05$).
Control: shoot tips subjected to pre-treatment with sucrose; LN^- = shoot tips exposed to cryoprotectants, but not immersed in liquid nitrogen; LN^+ = shoot tips subjected to all stages of cryopreservation.

Figure 1. Cryopreservation of *E. grandis* shoot tips. (A) 30-day-old seedlings of *E. grandis* (4.5 cm) as the source of cryopreserved shoot tips; (B, C) Regeneration of shoot tips in the control treatment after 30 and 60 days, respectively; (D, E, F) Regeneration of shoot tips exposed to the cryoprotectants PVS2, PVS3, and PVS4 (respectively) after 30 days of cryopreservation; (G) 60-day-old shoots after cryopreservation using the cryoprotectant PVS2; (H) Shoots in the elongation medium for 90 days, after cryopreservation using the cryoprotectant PVS2; (I) Mini-cuttings rooted in rooting medium after cryopreservation using PVS2 and subsequent exposure to shoot-elongation medium.

cryopreservation. Shoot tips that were treated with those two cryoprotectants showed high survival and regeneration rates as compared to those treated with PVS3, and even formed new rooted plants.

In addition to the chemical compositions of the cryoprotectants, the temperature maintained during their inoculation (0 °C) will also influence root tip protection. Low temperatures enhance the cryoprotectant efficiencies of solutions containing high toxicity components, such as PVS2, as low temperatures will reduce the speed at which the solution penetrates the cells and thus minimize its toxic effects. Ideal temperatures can vary, however, as slightly higher temperatures will favor the penetration of less toxic cryoprotectants and thus increase cell protection rates.

The efficiencies of the cryopreservation of PVS2 and PVS4 had been demonstrated in other species (Kaya et al. 2013, Bustam et al. 2016, Kaya and Souza 2017, Tahtamouni et al. 2017, Volk et al. 2018, Uchendu et al. 2019, Bettoni et al. 2019), with survival rates similar to, or higher than, those seen with *E. grandis*. A study using *Cannabis sativa* likewise evaluated the efficiency of PVS2, PVS3, and PVS4 as cryoprotectants, and the explants exposed to PVS3 showed the lowest regeneration percentages (Uchendu et al. 2019).

Most of the shoot tips in the present study that were exposed to PVS3 for 60 minutes, but not immersed in nitrogen (LN^-), survived and regenerated; it is therefore most probable that the shoot tips that had been immersed in liquid nitrogen (LN^+) did not survive due to negative effects of the very low temperature – and not because of some toxic effect of that cryoprotectant. That hypothesis could be tested by increasing the exposure time of the root apices to PVS3, and then assessing any improvements in its cryoprotective capacity.

In addition to optimizing some of the key parameters for cryopreservation success, this work was the first to use cryo-plates for the cryopreservation of eucalyptus. The cryo-plate method unites two different approaches with numerous advantages when compared to other vitrification techniques, including: reduced handling of the explants; faster procedures; explant protection via dehydration with cryoprotectants; cryopreservation of large numbers of samples simultaneously; and high survival and regeneration rates (Yamamoto et al. 2012, Engelmann 2014, Niino et al. 2014, Arizaga et al. 2017, Pettinelli et al. 2017).

A study using the cryo-plate technique with date palms revealed that, when combined with sucrose pre-treatment steps, the regrowth intensities of cryopreserved explants increased greatly, with rates close to 90% (Salma et al. 2014), as sucrose initiates the process of explant dehydration and thus avoids osmotic shocks and improves recovery after exposure to liquid nitrogen (Engelmann 2014, Salma et al. 2014). Additionally, cryopreservation using cryo-plates reduces direct contact of the explants with the vitrification solutions, as the alginate capsule formed around the explants helps protect them from direct exposure to those very toxic solutions (Sakai and Engelmann 2007, Pettinelli et al. 2017). The combination of those strategies makes this a viable cryopreservation alternative, especially for sensitive plants.

All the advantages cited above, together with the good survival and regeneration rates obtained with PVS2 and PVS4, make the cryo-plate methodology described in this work a viable alternative for the conservation of *E. grandis* germplasm. Our results also indicate ethylene glycol as a key compound in the success of cryopreservation techniques with eucalyptus shoot tips.

CONCLUSION

The cryo-plate technique was shown to be a viable methodology for the cryopreservation of *E. grandis* shoot tips, provided that adequate explant sources and cryoprotectants are used. Cryoprotectants PVS2 and PVS4 proved to be efficient in cryopreservation, with high percentages of regeneration and shoot production, as well as the subsequent formation of rooted mini-cuttings *in vitro*. Those high rates were associated with the cryoprotective capacity of ethylene glycol and its low toxicity.

REFERENCES

Ahn CH and Choi Ye (2017) In vitro clonal propagation and stable cryopreservation system for *Platycladus orientalis* via somatic embryogenesis. **Plant Cell, Tissue and Organ Culture 131**: 513-523.

Arizaga MV, Navarro OFV, Martinez CRC, Gutiérrez EJC, Delgado HAL, Yamamoto SI, Watanabe K and Niino T (2017) Improvement to the D cryo-plate protocol applied to practical cryopreservation of in vitro grown potato shoot tips. **The Horticulture Journal 86**: 222-228.

Bekheet SA, Sota V, El-Shabrawi HM and El-Ministy AM (2020) Cryopreservation of shoot apices and callus cultures of globe artichoke using vitrification method. **Journal of Genetic Engineering and Biotechnology 18**: 1-8.

Bettoni JC, Kretzschmar AA, Bonnart R, Sheperd A and Volk GM (2019) Cryopreservation of 12 *Vitis* species using apical shoot tips derived from plants grown in vitro. **Hortscience 54**: 976-981.

Brison M, Boucaud MT and Dosba F (1995) Cryopreservation of in vitro grown shoot tips of two interspecific *Prunus* rootstocks. **Plant Science 105**: 235-242.

Bustam BM, Dixon K and Bunn E (2016) A cryopreservation protocol for ex situ conservation of terrestrial orchids using asymbiotic primary and secondary (adventitious) protocorms. **In Vitro Cellular and Developmental Biology – Plant 52**: 185-195.

Carneros E, Hernández I, Toribio M, Díaz-Sala C and Celestino C (2017) Effect of different cryoprotectant procedures on the recovery and maturation ability of cryopreserved *Pinus pinea* embryogenic lines of different ages. **In Vitro Cellular & Developmental Biology – Plant 53**: 469-477.

Dixit S, Ahuja S, Narula A and Srivastava PS (2005) Cryopreservation: A potential tool for long-term conservation of medicinal plants. In Srivastava PS, Narula A, Srivastava S (eds) **Plant biotechnology and molecular markers**. Springer, New Delhi, p. 278-288.

Ellis D, Skogerboe D, Andre C, Hellier B and Volk G (2006) Implementation of garlic cryopreservation techniques in the national plant germplasm system. **Cryo Letters 27**: 99-106.

Engelmann F (1992) Effects of freezing in liquid nitrogen on the properties of a soybean (*Glycine max* L. var. acme) callus strain used as a bioassay for cytokinin activity. **Cryo Letters 13**: 331-336.

Engelmann F (2004) Cryopreservation of plant species: progress and prospects. **In Vitro Cellular and Developmental Biology – Plant 40**: 427-433.

Engelmann F (2014) Cryopreservation of clonal crops: a review of key parameters. **Acta Horticulturae 1039**: 31-39.

Fabre J and Dereuddre J (1990) Encapsulation dehydration: a new approach to cryopreservation of Solanum shoot-tips. **Cryo Letters 11**: 413-426.

Ferreira DF (2008) SISVAR: A software for analysis and teaching statistics.

Symposium 6: 36-41.

Funnekotter B, Mancera RL and Bunn E (2017) Advances in understanding the fundamental aspects required for successful cryopreservation of Australian flora. **In Vitro Cellular and Developmental Biology – Plant 53**: 289-298.

Gonçalves JLM, Alvares CA, Higa AR, Silva LD, Alfenas AC, Stahl J, Ferraz SFB, Lima WP, Brancalion PHS, Hubner A, Bouillet JD, Laclau J, Nouvellon Y and Epron D (2013) Integrating genetic and silvicultural strategies to minimize abiotic and biotic constraints in Brazilian eucalypt plantations. **Forest Ecology and Management 301**: 6-27.

Grattapaglia D and Kirst M (2008) Eucalyptus applied genomics: from gene sequences to breeding tools. **New Phytologist 179**: 911-929.

Gross BL, Henk AD, Bonnart R and Volk GM (2017) Changes in transcript expression patterns as a result of cryoprotectant treatment and liquid nitrogen exposure in Arabidopsis shoot tips. **Plant Cell Reports 36**: 459-470.

Gupta S (2014) Cryopreservation of germplasm through encapsulation-dehydration technique. **Acta Horticulturae 1039**: 147-153.

Kaya E and Souza FVD (2017) Comparison of two PVS2-based procedures for cryopreservation of commercial sugarcane (*Saccharum* spp.) germplasm and confirmation of genetic stability after cryopreservation using ISSR markers. **In Vitro Cellular and Developmental Biology – Plant 53**: 410-417.

Kaya E, Alves A, Rodrigues L, Jenderek M, Hernandez-Ellis M, Ozudogru A and Ellis D (2013) Cryopreservation of Eucalyptus genetic resources. **Cryo Letters 34**: 608-618.

Kim HH, Lee YG, Park SU, Lee SC, Baek HJ, Cho EG and Engelmann F (2009) Development of alternative loading solutions in droplet-vitrification procedures. **Cryo Letters 30**: 291-299.

Kreck CA, Mandumpal JB and Mancera RL (2011) Prediction of the glass transition in aqueous solutions of simple amides by molecular dynamics simulations. **Chemical Physics Letters 501**: 273-277.

Li JW, Ozudogru EA, Li J, Wang MR, Bi WL, Lambardi M and Wang QC (2018) Cryobiotechnology of forest trees: recent advances and future prospects. **Biodiversity and Conservation 27**: 795-814.

Lloyd G and McCown B (1981) Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot tip culture. **International Plant Propagation Society Proceedings 30**: 421-327.

Malajczuk CJ, Hughes ZE and Mancera RL (2013) Molecular dynamics simulations of the interactions of DMSO, mono-and polyhydroxylated cryosolvents with a hydrated phospholipid bilayer. **Biochimica et Biophysica Acta (BBA) - Biomembranes 1828**: 2041-2055.

Melo CG, Barbosa MHP, Motoike SY, Sabino MV, Ventrella MC, Peternelli LA and Oliveira MAR (2011) Preculture sugarcane tissue in sucrose supplemented culture medium to induce desiccation tolerance. **Crop Breeding and Applied Biotechnology 11**: 320-329.

Murashige T and Skoog F (1962) A revised medium for rapid growth and bio-assays with tobacco tissue culture. **Physiologia Plantarum 15**: 473-497.

Nadarajan J and Pritchard HW (2014) Biophysical characteristics of successful oilseed embryo cryoprotection and cryopreservation using vacuum infiltration vitrification: an innovation in plant cell preservation. **Plos One 9**: e96169.

Niino T, Watanabe K, Nohara N, Rafique T, Yamamoto SI, Fukui K, Arizaga MV, Martinez CRC, Matsumoto T and Engelmann F (2014) Cryopreservation of mat rush lateral buds by air dehydration using aluminum cryo-plate. **Plant Biotechnology 3**: 281-287.

Nishizawa S, Sakai A, Amano Y and Matsuzawa T (1993) Cryopreservation of asparagus (*Asparagus officinalis* L.) embryogenic suspension cells and subsequent plant regeneration by vitrification. **Plant Science 91**: 67-73.

Oliveira ATD, Forell F, Medeiros CMO, Lopes RFF and Rodrigues EJL (2003) Vitrification of IVMFC-Derived bovine embryos using ethylene glycol and sucrose. **Ars Veterinaria 19**: 191-201.

Padayachee K, Watt MP, Edwards N and Mycock DJ (2009) Cryopreservation as a tool for the conservation of Eucalyptus genetic variability: concepts and challenges. **Southern Forests: A Journal of Forest Science 71**: 165-170.

Panis B, Piette B and Swennen R (2005) Droplet vitrification of apical meristems: a cryopreservation protocol applicable to all Musaceae. **Plant Science 168**: 45-55.

Pettinelli JA, Soares BO, Cantelmo L, Garcia RO, Mansur E, Engelmann F and Gagliardi RF (2017) Cryopreservation of somatic embryos from *Petiveria alliacea* L. by different techniques based on vitrification. **In Vitro Cellular and Developmental Biology – Plant 53**: 339-345.

Pukacki PM and Juszczyk K (2015) Desiccation sensitivity and cryopreservation of the embryonic axes of the seeds of two Acer species. **Trees 29**: 385-396.

Rao NK (2004) Plant genetic resources: Advancing conservation and use through biotechnology. **African Journal of Biotechnology 3**: 136-145.

Sakai A (2000) Development of cryopreservation techniques. In Engelmann F and Takagi H (eds) **Cryopresevation of tropical plant germplasm, current progress and application**. IPGRI, Rome, p. 1-7.

Sakai A and Engelmann F (2007) Vitrification, encapsulation-vitrification and droplet-vitrification: a review. **Cryo Letters 28**: 151-172.

Sakai A, Kobayashi S and Oiyama I (1990) Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb. var. brasiliensis Tanaka) by vitrification. **Plant Cell Reports 9**: 30-33.

Salma M, Fki L, Engelmann-Sylvestre I, Niino T and Engelmann F (2014) Comparison of droplet-vitrification and D-cryoplate for cryopreservation of date palm (*Phoenix dactylifera* L.) polyembryonic masses. **Scientia Horticulturae 179**: 91-97.

Tahtamouni, RW, Shibli RA, Al-Abdallat AM, Al, Qudah TS, Younis L, Albaba H and Al-Ruwaiei H (2017) Cryopreservation of *Thymbra spicata* L. var. spicata and genetic stability assessment of the cryopreserved shoot tips after conservation. **Jordan Journal of Biological Sciences**

10: 19-28

Tao D and Li PH (1986) Classification of plant cell cryoprotectants. **Journal of Theoretical Biology 123**: 305-310.

Trueman SJ, Hung CD and Wendling I (2018) Tissue culture of Corymbia and Eucalyptus. **Forests 9**: 1-42.

Turner S, Senaratna T, Touchell D, Bunn E, Dixon K and Tan B (2001) Stereochemical arrangement of hydroxyl groups in sugar and polyalcohol molecules as an important factor in effective cryopreservation. **Plant Science 160**: 489-497.

Uchendu E, Lata H, Chandra S, Khan IA and ElSohly MA (2019) Cryopreservation of shoot tips of elite cultivars of *Cannabis sativa* L. by Droplet Vitrification. **Medical Cannabis and Cannabinoids 2**: 29-34.

Uchendu EE and Reed BM (2008) A comparative study of three cryopreservation protocols effective storage of in vitro-grown mint (*Mentha* spp.). **Cryo Letters 29**: 81-188.

Volk GM and Caspersen AM (2017) Cryoprotectants and components induce plasmolytic responses in sweet potato (*Ipomoea batatas* (L.) Lam.) suspension cells. **In Vitro Cellular & Developmental Biology – Plant 53**: 363-371.

Volk GM and Walters C (2006) Plant vitrification solution 2 lowers water content and alters freezing behaviour in shoot tips during cryoprotection. **Cryobiology 52**: 48-61.

Volk GM, Harris JL and Rotindo KE (2006) Survival of mint shoot tips after exposure to cryoprotectant solution components. **Cryobiology 52**: 305-308.

Volk GM, Shepherd AM and Bonnart R (2018) Successful cryopreservation of Vitis shoot tips: novel pre-treatment combinations applied to nine species. **Cryo Letters 39**: 322-330.

Yamamoto S, Rafique T, Fukui K, Sekizawa K and Niino T (2012) V-cryo-plate procedure as an effective protocol for cryobanks: case study of mint cryopreservation. **Cryo Letters 33**: 12-23.

Yamamoto S, Rafique T, Priyantha WS, Fukui K, Matsumoto T and Niino T (2011) Development of a cryopreservation procedure using aluminium cryo-plates. **Cryo Letters 32**: 256-265.