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Cryopreservation of *Dendrobium* sonia-28 using an alternative method of PVS2 droplet freezing

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Abstract

Dendrobium sonia-28, an ornamental orchid in the Malaysian flower industry, is under risk of producing heterozygous progenies. Cryopreservation is a favoured long-term storage method for orchids with propagation problems. The droplet-freezing technique, utilising the principles of vitrification, has emerged as an important technique in the preservation of various plant species. This study was conducted to optimise the droplet-freezing technique for protocorm-like bodies (PLBs) of *Dendrobium* sonia-28, with viability tests conducted through the 2,3,5-triphenyltetrazolium chloride assay. The best results were obtained when 1-2 mm PLBs were precultured for 24 hours on half-strength Murashige and Skoog (MS) semi-solid medium supplemented with 0.5 M sucrose, placed in a loading solution for 10 minutes and dehydrated in PVS2 at 0°C for 20 minutes, prior to thawing, unloading in 1.2 M sucrose and recovery on semi-solid recovery medium composed of half-strength MS components and 2% sucrose for three weeks.

Key words: *Dendrobium* sonia-28, PVS2 droplet-freezing, Protocorm-like bodies

Introduction

Commercially valuable *Dendrobium* orchids such as *Dendrobium* sonia-28 are popular as cut flowers and ornamental plants due to their frequent flowering and high number of flowers for each inflorescence (Martin and Madassery, 2006). *Dendrobium* wholesale in Hawaii is valued at about USD 10 million (Sewake, 1999), while *Dendrobium* cut flowers contributed 80% of total cut flower production in Thailand (Bureau of Agricultural Economics Research, 2003; Samtinoranont and Wannakrairoj, 2010). The economic importance of *Dendrobium* orchids increases the urgency to conserve valuable orchid germplasm. The need of orchid conservation is also driven by various factors such as loss of orchid habitat in nature and illegal collection by hobbyist (Siregar, 2008).

Cryopreservation presently provides a long term conservation option, requiring only a minimum of space and maintenance (Sakai et al., 1991). For successful cryopreservation, intracellular freezing, which occurs during rapid cooling in liquid nitrogen (LN), must be avoided. One of the keys to successful cryopreservation by vitrification is the careful control of dehydration and prevention of injury by chemical toxicity. Specimens to be preserved have to be sufficiently dehydrated to avoid intracellular freezing and thus vitrify upon rapid cooling (Pandey et al., 2008).

The droplet-vitrification method, reported to be a promising technique in the plant cryopreservation field, is based on the droplet-freezing method that was established for potato (Schäfer-Menuhr et al., 1997) using 10% DMSO as the cryoprotectant. Droplet-vitrification is a cryopreservation method capitalising on the direct vitrification of small volumes of water containing cryoprotective additives placed on highly efficient, heat-conducting materials such as aluminium foil (Panis et al., 2005; Sakai and Engelmann, 2007; Galdiano Jr. et al, 2012). Successful attempts of this method have been reported for asparagus (Mix-Wagner et al., 2000), yam (Leunufna and Keller, 2003) and rose (Halmagyi and Pinker, 2006). Highly concentrated cryoprotectants such as plant vitrification solution 2 (PVS2) are used to protect the cells during freezing and to promote recovery of the plant tissue after storage in liquid nitrogen

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(Sakai, 1997). The droplet-vitrification method was the only method giving regeneration in three banana cultivars: Nakitengwa, Ingarama and Amou (Panis et al., 2004).

A post-cryopreservation viability test is defined as a test "that evaluates stress caused to plant tissue by cryopreservation and expresses the probability for survival or regrowth" (Verleysen at al., 2004). The best survival assessment of cryopreserved tissues is through regrowth of the target tissues into plantlets. However, regrowth is very slow in many cases, especially when a cryopreservation protocol is still in the optimisation stage. In this case, application of viability tests, based on the cellular absorption of dyes cannot be avoided, as most cryopreservation protocols require rapid assessments on whether the material survived the cryopreservation procedure (Leslie et al., 1995). The TTC assay, qualitative for large tissues and organs, is often used for embryos and axes, and is reduced into red-coloured formazan by respiration in mitochondria of the living cells. This method is based on the metabolic activity of living plant cells, as living cells or viable areas of the target plant sample absorbs the colourless TTC compound, which is then reduced by dehydrogenases, yielding triphenylformazan or reduced TTC (Van Waes and Debergh, 1986; Verleysen at al., 2004). Tsukazaki et al. (2000) reported that the TTC reduction assay and regrowth observations used in the assessment of survival of *Doritaenopsis* suspension culture cryopreserved by vitrification were correlated.

In this study, the plant vitrification solution 2, commonly known as PVS2 (Sakai et al., 1991) was used as a cryoprotectant to dehydrate and cryopreserve protocorm-like bodies (PLBs) of *Dendrobium* sonia-28 via the droplet-freezing method. The objectives of the present study are to ascertain the best PLBs size suitable for cryopreservation, and also to evaluate the effects of various sucrose concentrations, loading periods and PVS2 treatment periods on the viability of cryopreserved PLBs of *Dendrobium* sonia-28.

Materials and Methods

Plant material

In vitro cultures of protocorm-like bodies (PLBs) of *Dendrobium* sonia-28 were selected for this study. The PLBs were cultured on half-strength Murashige and Skoog (MS; Murashige and Skoog, 1962) semi-solid media supplemented with 1 mg/L benzylaminopurine (BAP), 20 g/L sucrose and 2.75 g/L Gelrite. The cultures were incubated at 25±2ºC under 16-hours photoperiod using white fluorescent lamps (Philips TLD, 36 W) set at 150 µmol m^{-2} s^{-1}. The PLBs were subcultured every four weeks.

Effect of PLB size

In order to determine the best PLB size in the application of the droplet-freezing method, four-week old 1-2 and 3-4 mm PLBs of *Dendrobium* sonia-28 were precultured on medium consisting of semi-solid half-strength MS components and 0.5 M sucrose. The PLBs were then incubated for 24 hours at 25±2ºC under 16 hours photoperiod using white fluorescent lamps (Philips TLD, 36 W) set at 150 µmol m^{-2} s^{-1}. The PLBs were then placed in sterile cryovials and immersed with a loading solution composed of liquid half-strength MS medium supplemented with 0.4 M sucrose and 2.0 M glycerol, for 20 minutes. For dehydration, the PLBs were immersed in PVS2 (30% [w/v] glycerol, 15% [w/v] ethylene glycol, 15% [w/v] Me_2SO and 0.4 M sucrose at 0ºC for 20 minutes (Sakai et al., 1991). The PVS2 was drained from the cryovials and the PLBs were then transferred onto 10 µl PVS2 droplets placed on a strip of sterile aluminium foil. The foils were carefully folded and submerged into LN for at least one hour. All solutions used were exchanged with fresh solutions after 10 minutes.

Effect of preculture

To determine the best sucrose concentration for preculture, PLBs with the optimal size (as selected from the previous treatment) were cultured on half-strength MS semi-solid medium supplemented with various sucrose concentration (0.0, 0.25, 0.5, 0.75, and 1.0 M). The osmoprotection and dehydration steps were conducted as in the determination of the best PLB size for cryopreservation.

Effect of loading and dehydration treatments

The best PLB size and preculture conditions were selected from the previous sections to proceed with the next steps of the treatment. In order to assess the effect of loading duration on the PLBs, the PLBs were immersed in loading solution for 10, 15, 20, 25, 30 and 35 minutes, with an exchange of fresh solution at half-time intervals. This was followed by immersing the PLBs in 1.5 ml PVS2 for 20 minutes at 0°C. The solution was exchanged after 10 minutes with fresh PVS2.

In order to assess the effect of dehydration on the PLBs, the PLBs were immersed in 1.5 ml loading solution (half-strength MS medium supplemented with 2 M glycerol and 0.4 M sucrose) at room temperature for 20 minutes. The loading solution was removed and replaced with 1.5 ml PVS2 at 0°C for 10, 20, 30 or 40 minutes.

Thawing, unloading and PLBs growth recovery

Non-cryopreserved and cryopreserved PLBs from all treatments were subjected to the recovery steps mentioned in this section. The foils were carefully removed from LN and rapidly thawed in 10 ml unloading solution (half-strength MS solution with 1.2 M sucrose) for 20 minutes at room temperature. After 20 minutes, the PLBs were transferred onto a piece of sterile filter paper to blot dry the unloading solution.

The PLBs were transferred onto two pieces of sterile filter papers placed on semi-solid recovery medium composed of half-strength MS components and 2% sucrose, and incubated in the dark for one day at 25±2ºC. The PLBs were then transferred onto fresh recovery medium without the filter paper and incubated in the dark at 25±2ºC for one week. The PLBs were slowly exposed to dim light by incubation under a layer of white cloth for one week. Finally, the PLBs were exposed to light and incubated for one more week at 25±2ºC under 16 hours photoperiod using white fluorescent lamps (Philips TLD, 36 W) set at 150 µmol m^{-2} s^{-1}. Non-cryopreserved PLBs were subjected to the same treatment as cryopreserved PLBs, except for storage in LN.

PLBs viability assessment

After 3 weeks of recovery, the viability of cryopreserved and non-cryopreserved PLBs were assessed using the 2,3,5-triphenyltetrazolium chloride (TTC) analysis, set at 490nm (Verleysen et al., 2004).

Statistical Analyses

The treatments consisted of six replicates, each containing 10 PLBs. Means in the determination of the best PLB size were analysed through independent sample's t-test. Means from other treatments were analysed with one-way ANOVA and differentiated with Tukey's test. The probability value was set at 0.05.

Results

Effect of PLB size

The results of this study showed that 1-2 mm cryopreserved PLBs produced higher absorbance values compared to 3-4 mm cryopreserved PLBs (Figure 1), hence their selection for the subsequent steps of the experiment. On the other hand, 3-4 mm PLBs produced higher absorbance values compared to 1-2 mm PLBs in the control treatments.

Effect of Sucrose Preculture

It was observed that the cellular viability of cryopreserved and non-cryopreserved PLBs was the highest when PLBs were precultured in 0.5 M sucrose (Figure 2). The viability of cryopreserved PLBs decreased when they were precultured in higher sucrose concentrations (>0.5 M) prior to storage in LN.

Effect of loading treatment

Results from the loading treatment showed that 10 minutes of loading produced the highest viability for cryopreserved PLBs of *D.* sonia-28 (Figure 3). The decrease in cellular viability after the 10th minute indicated that extended exposure to loading solution is harmful for PLBs of *D.* sonia-28.

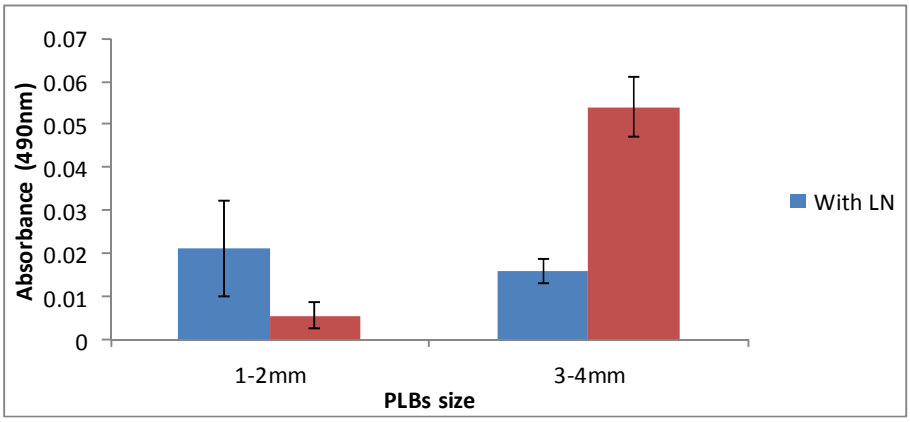

Figure 1. Effect of PLB size in the cryopreservation of *Dendrobium* sonia-28. The PLBs were precultured in 0.5 M sucrose prior to loading, dehydration and storage in LN. The PLB sizes tested were 1-2 mm and 3-4 mm. Error bars show corresponding standard deviation.

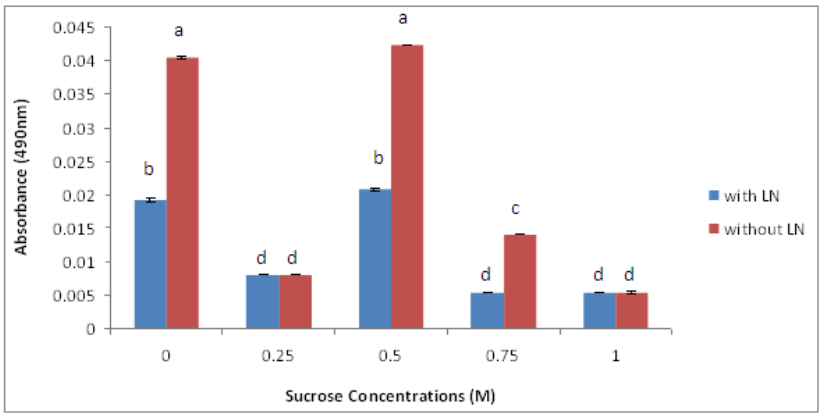

Figure 2. Effect of preculture on the viability of non-cryopreserved and cryopreserved PLBs of *Dendrobium* sonia-28. The tested sucrose concentrations were 0, 0.25, 0.50, 0.75 and 1.0 M. Error bars show corresponding standard deviation.

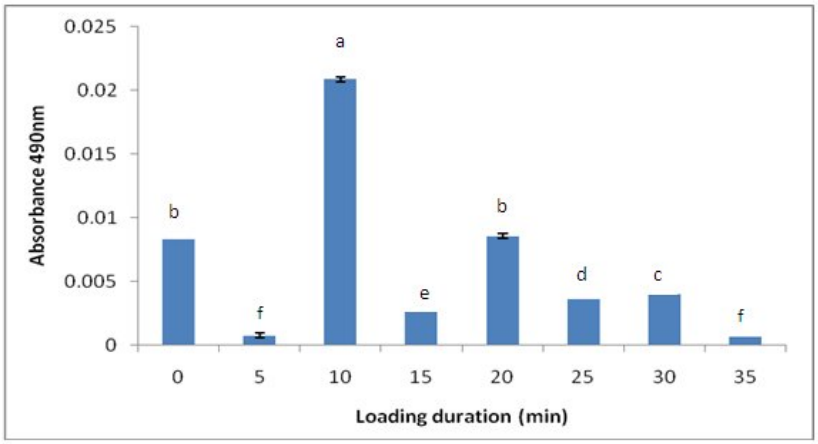

Figure 3. Effect of different loading durations on the viability of cryopreserved PLBs of *Dendrobium* sonia-28. The tested loading durations were 0, 5, 10, 15, 20, 30 and 35 minutes. Error bars show corresponding standard deviation.

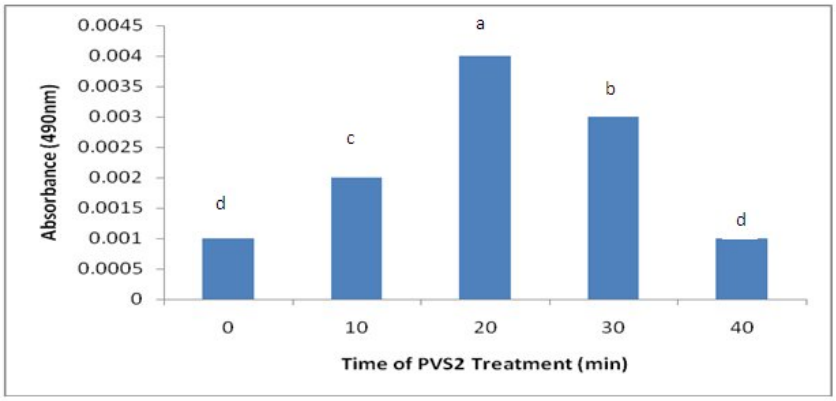

Figure 4. Effect of different dehydration durations on the viability of cryopreserved PLBs of *Dendrobium* sonia-28. The tested PVS2 durations were 0, 10, 20, 30, and 40 minutes. Error bars show corresponding standard deviation.

Effect of PVS2 treatment

Cryopreserved PLBs of *D*. sonia-28 showed increasing cellular viability with increased exposure to PVS2 (Figure 4). A significantly high cellular viability was recorded when PLBs were exposed for 20 minutes to PVS2, suggesting that the PLBs can be effectively dehydrated at the mentioned duration without causing harmful effect to the cells. The drastic decline of cellular viability in PLBs of *D*. sonia-28, with increasing exposure to PVS2 (30 and 40 minutes), could be due to the toxic effect of the solution.

Discussion

Effect of PLB size

The best results in the cryopreservation of PLBs of *Dendrobium* sonia-28 were obtained when 1-2 mm PLBs were used in the vitrification protocol, compared to 3-4 mm PLBs. This result corresponds to that obtained by Pouzi et al. (2011), who discovered that the best viability in the encapsulation-dehydration of *D.* sonia-28 was obtained when 1-2 mm PLBs were precultured in 1.0 M sucrose for 24 hours (Pouzi et al., 2011). In *Iris nigricans*, 2–4 mm somatic embryos produced higher survival rates compared to 1–2 mm or 4–6 mm embryos (Shibli, 2000; Lambardi et al., 2008). Lambardi et al. (2005) discovered that within the callus clumps of *Aesculus hippocastanum*, embryogenic masses at an advanced stage of somatic embryo maturation, for instance, the torpedo stage, produced optimum post-cryopreservation regrowth of healthy and proliferating embryogenic callus, performing better than callus clumps composed predominantly of globular, heart shaped and cotyledonary somatic embryos (Lambardi et al., 2008). Hence, the outcome of this cryopreservation treatment could have depended on the age or the growth stage of the PLBs prior to the cryopreservation process.

Effect of Sucrose Preculture

High sucrose concentrations applied in this study resulted in excessive cell dehydration, with consequences on the viability of explants (Halmagyi and Pinker, 2006). The cellular viability of the PLBs in this study displayed a similar pattern to that observed in cryopreserved *Lilium* (Chen et al., 2010). The best results in the encapsulation-vitrification of PLBs of *Dendrobium candidum* Wall. ex Lindl. were obtained when the PLBs were subjected to a five-day preculture in 0.75 M sucrose medium with BAP and 1-naphthaleneacetic acid (NAA) (Yin and Hong, 2009). Results of a study by Tan et al. (2010) showed that the best viability rate in the cryopreservation of *D.* sonia-28 was achieved when 3-4 mm PLBs were precultured in semi-solid half-strength MS media with 0.6 M sucrose and dehydrated in PVS2 at 0°C for 20 minutes. In the cryopreservation of *Dendrobium* Bobby Messina by vitrification, the best viability rate was obtained when 3-4 mm PLBs were precultured in either 0.6 M sucrose or 1.2 M sorbitol (Antony et al., 2010).

Preculture of plant materials in medium containing sucrose or sorbitol boosted the survival of cryopreserved shoot tips of white clover (Yamada et al., 1991), apple and pear (Niino et al., 1992). The beneficial effect of sucrose in cryopreservation could be due to two effects (Steponkus et al., 1992). First, sucrose, like other osmotically active substances, has an osmotic dehydration effect during treatment, leading to reduced water content in the tissue (Reinhoud et al., 1995, Tanaka et al., 2004). Furthermore, sucrose is able to penetrate the cells (Dumet et al., 1993), proven by histological observations of intracellular accumulation of starch during preculture (González-Arnao et al., 1998). The accumulation of sucrose within the tissue contributes to the cell viability by assisting in the removal of cellular water to the point of glassy state during vitrification (Steponkus et al., 1992). Sugars are also known to play a crucial role in the preservation of the membrane integrity (Crowe et al., 1988) and protein structure (Leslie et al., 1995) during dehydration.

Effect of loading treatment

The loading solution is credited in the reduction of injurious membrane changes resulting from severe dehydration (Ishikawa et al., 1997). In this study, the effect of the loading solution on the survival of PLBs of *Dendrobium* sonia-28 was neither readily apparent nor significant in either the control or freezing treatments. Wang et al. (2004) stated that the loading treatment, regardless of the immersion durations, did not influence the viability of grapevine embryogenic cell suspensions, with viability percentages recorded at about 85 and 76% for non-cryopreserved and cryopreserved cells respectively. Panis et al. (2005) also observed that loading treatment conducted for any duration did not significantly affect regeneration of control or cryopreserved *Musaceae* meristems. However, a study by Takagi et al. (1997) showed that a loading treatment for taro shoot tips was important in ensuring their survival post-cryopreservation, with

the best result obtained when the samples were osmoprotected for 20 minutes.

Effect of PVS2 treatment

Cryoprotectants such as PVS2 function to protect and to recover the plant material after storage in LN (Sakai, 1997). The optimal dehydration treatment of different plant species (with varying water content and membrane permeability) can differ considerably. Towill and Jarret (1992), and Sakai (1997) reported that long exposure of explants to highly concentrated vitrification solution is potentially injurious because of the phytotoxic effects of individual component or combined osmotic effect on cell viability.

Tiau et al. (2009) reported that 2-4 mm PLBs of *D.* sonia-28 gave the highest viability when PLBs were precultured in 0.25 M sucrose, followed by dehydration in PVS2 at 0°C for 20 minutes. This corresponds to the results obtained in this study. Cryopreserved PLBs of *Bratonia* had to be dehydrated for one hour in modified PVS2 supplemented with PEG instead of ethylene glycol to boost post-thawing recovery percentages up to 20.4% (Popova et al., 2010). Tsukazaki et al. (2000) discovered that the use of PVS2 was detrimental to the survival of *Doritaenopsis* suspension culture as the TTC stainability reduced to 80% when the cells were precultured in 0.056, 0.1, 0.2, 0.3 or 0.4M sucrose and immersed in PVS2, compared to cells that were simply precultured and not dehydrated (85%). However, cells that were not dehydrated did not survive the cryopreservation procedure at all. A three- to five-hour dehydration period was required for the embryos of *Bletilla striata* (Ishikawa et al., 1997). On the other hand, apical meristems of Japanese horseradish required only 10 minutes of dehydration in PVS2 prior to cryostorage (Matsumoto et al., 1994). The differences in the dehydration period for various plant species was attributed to the cell clump sizes, developmental stages and physiological condition of the explant (Tsukazaki et al., 2000).

Conclusion

In this study, it was found that the highest viability in the cryopreservation of PLBs of *Dendrobium* sonia-28 through the droplet-freezing method was achieved when 1-2 mm PLBs were precultured in half-strength MS medium supplemented with 0.5 M sucrose, placed in the loading solution for 10 minutes, and dehydrated in PVS2 for 20 minutes.

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References

Antony, J. J. J., L. K. Chan, R. Xavier R, S. Uma Rani and S. Sreeramanan. 2010. Preliminary study on cryopreservation of *Dendrobium* Bobby Messina protocorm-like bodies by vitrification. Afr. J. Biotechnol. 9(42):7063-7070.

Bureau of Agricultural Economics Research. 2003. Orchid production and marketing. Office of Agricultural Economic, Ministry of Agriculture and Cooperatives. p. 64.

Chen, X. L., J. H. Li, X. Xin, Z. E. Zhang, P. P. Xin and X. X. Lu. 2011. Cryopreservation of *in vitro*-grown apical meristems of *Lilium* by droplet-vitrification. S. Afr. J. Bot. 77(2):397–403.

Crowe, J. H., L. M. Crowe, J. F. Carpenter, A. S. Rudolph, C. A. Wistrom, B. J. Spargo and T. J. Anchordoguy. 1988. Interactions of sugars with membranes. Biochim. Biophys. Acta 947:367-384.

Dumet, D., F. Engelmann, N. Chabrillange, Y. Duval and J. Dereuddre. 1993. Importance of sucrose for the acquisition of tolerance to desiccation and cryopreservation of oil palm somatic embryos. Cryo Lett. 14:243-250.

Galdiano Jr., R. F., E. G. M. Lemos, R. T. Fari and W. A. Vendrame. 2012. Cryopreservation of *Dendrobium* hybrid seeds and protocorms as affected by phloroglucinol and Supercool X1000. Sci. Hort. 148:154–160

González-Arnao, M. T., M. M. Ravelo, C. U. Villavicencio, M. M. Montero and F. Engelmann. 1998. Cryopreservation of pineapple (*Ananas comosus*) apices. Cryo Lett. 19:375-382.

Halmagyi, A. and I. Pinker. 2006. Plant regeneration from *Rosa* shoot tips cryopreserved by a combined droplet-vitrification method. Plant Cell Tiss. Org. 84:145–153.

Heine-Dobbernack, E., H. Kiesecker and H. M. Schumacher. 2008. Cryopreservation of Dedifferentiated Cell Cultures. In: Reed, B. M. (ed.). Plant Cryopreservation: A Practical Guide. Springer. pp. 141-176.

Ishikawa, K, K. Harata, M. Mii, A. Sakai, K. Yoshimatsu and K. Shimomura. 1997. Cryopreservation of zygotic embryos of a Japanese terrestrial orchid (*Bletilla striata*) by vitrification. Plant Cell Rep. 16:754–757.

Leslie, S. B., E. Israeli, B. Lighthart, J. H. Crowe and L. M. Crowe. 1995. Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying. Appl. Environ. Microbiol. 61:3592-3597.

Leunufna, S. and E. R. J. Keller. 2003. Investigating a new cryopreservation protocol for yams (*Dioscorea* spp.). Plant Cell Rep. 21:1159–1166.

Martin, K. P. and J. Madassery. 2006. Rapid *in vitro* propagation of *Dendrobium* hybrids through direct shoot formation from foliar explants and protocorm-like bodies. Sci. Hort. 108:95-99.

Matsumoto, T., A. Sakai and K. Yamada. 1994. Cryopreservation of *in vitro*-grown apical meristems of wasabi (*Wasabia japonica*) by vitrification and subsequent high plant regeneration. Plant Cell Rep. 13(8):442-446.

Mikula, A. M. Niedzielski and J. J. Rybczynski. 2006. The use of TTC reduction assay for assessment of *Gentiana spp.* cell suspension viability after cryopreservation. Acta Physiol. Plant. 28(4):315-324.

Mix-Wagner, G., A. J. Conner and R. J. Cross. 2000. Survival and recovery of asparagus shoot tips after cryopreservation using the 'droplet' method. N. Z. J. Crop Hort. Sci. 28:283–287.

Murashige, T. and F. Skoog. 1962. A Revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 15:473-497.

Niino, T., A. Sakai, H. Yakuwa and K. Nojiri. 1992. Cryopreservation of *in vitro* grown shoot tips of apple and pear by vitrification. Plant Cell Tiss. Org.. 28:261-266.

Normah, M. N. and A. M. Makeen. 2008. Cryopreservation of Excised Embryos and Embryonic Axes. In: B. M. Reed (ed.), *Plant Cryopreservation: A Practical Guide*, pp. 211-240, Springer Science+Business Media, LLC.

Pandey, R., N. Sharma and R. Chamola. 2008. Cryoprotectant solutions and pretreatment media for cryopreservation. *In:* Laboratory Manual for *In Vitro* Conservation and Cryopreservation Techniques for Conservation of Plant Genetic Resources, 3rd Edition.

Panis, B., K. Vandenbranden, H. Schoofs and R. Swennen. 1998. Conservation of banana germplasm through cryopreservation. In: R. A. Drew (ed.) pp. 515-521. Proceedings of the International Symposium on Biotechnology of Tropical and Subtropical Species, Brisbane, Queensland, Australia.

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

Popova, E., N. Bukhov, A. Popov and H. H. Kim. 2010. Cryopreservation of protocorm-like bodies of the hybrid orchid *Bratonia* (*Miltonia flavescens* × *Brassia longissima*). Cryo Letters 1(5):426-437.

Reinhound, P. J., E. W.M. Schrijnemakers, F. van Iren and J. W. Kijne. 1995. Vitrification and a heat-shock treatment improve cryopreservation of tobacco cell suspensions compared to two-step freezing. Plant Cell Tiss. Org. 42:261-267.

Sakai, A. 1997. Potentially Valuable Cryogenic Procedures for Cryopreservation of Cultured Plant Meristem. In: M. K. Razdan and E. C. Cocking (Eds.). pp.53-66. Conservation of Plant Genetic Resources *in vitro* General Aspects, Science Publishers Inc., Enfield, NH.

Sakai, A. and F. Engelmann. 2007. Vitrification, encapsulation-vitrification and droplet vitrification: a review. Cryo Lett. 28:151–172.

Sakai, A., S. Kobayashi and L. Oiyama. 1991. Survival by vitrification of nucellar cells of navel orange (*Citrus sinensis var. brasiliensis* Tanaka) cooled to -196°C. J. Plant Physiol. 137:465-470.

Sarntinoranont, V. and S. Wannakrairoj. 2010. The relationship between environmental factors during rainy season and un-opened floret yellowing in *Dendrobium* sonia 'Ear-sakul'. Kasetsart J. (Nat. Sci.) 44:1016-1025.

Schäfer-Menuhr, A., G. Mix-Wagner and H. M. Schumacher. 1997. Cryopreservation of potato cultivars-design of a method for routine application in gene banks. Acta Hort. 447:477-483.

Sewake, K. 1999. Growing *Dendrobium* orchid in Hawaii. Production and pest management guide: Introduction. Leonhardt K., and K. Sewake (eds.). College of Tropical Agriculture and Human Resources. University of Hawaii, Manoa. p. 96.

Siregar, C. 2008. Exploration and inventory of native orchid germplasm in West Borneo, Indonesia. HortSci. 43(2):554-557.

Steponkus, P. L., R. Langis and S. Fujikawa S. 1992. Cryopreservation of plant tissues by vitrification. In: Advances in Low Temperature Biology (Vol. 1). Steponkus, P.L. (ed.). JAI Press Ltd. p. 1-61.

Takagi, H., N. Tien Thinh, O. M. Islam, T. Senboku and A. Sakai. 1997. Cryopreservation of *in vitro*-grown shoot tips of taro (*Colocasia esculenta* (L.) Schott) by vitrification. 1. Investigation of basic conditions of the vitrification procedure. Plant Cell Rep. 16(9):594-599.

Tan, H. H., J. James, J. Advina, P. Ranjeeta, G. Pavallekoodi and S. Sreeramanan. 2010. A Novel Approach for Preliminary PVS2 Vitrification Optimization Parameters of *Dendrobium* Sonia-28 Orchid with Evan Blue Staining. Adv. Environ. Biol. 4(2):284-290.

Tanaka, D., T. Niino, K. Isuzugawa, T. Hikage and M. Uemura. 2004. Cryopreservation of shoot apices of *in vitro* grown gentian plants: Comparison of vitrification and encapsulation-vitrification protocols. Cryo Lett. 25:167-176.

Tiau, K. H., R. Xavier, L. K. Chan and S. Sreeramanan. 2009. An Assessment of Early Factors Influencing the PVS2 Vitrification Method Using Protocorm-like Bodies of *Dendrobium* Sonia 28. Am.-Eurasian J. Sustain. Agric. 3(3):280-289.

Towill, L. E. and R. L. Jarret. 1992. Cryopreservation of sweet potato (*Ipomea batatas* (L.) Lam.) shoot tips by vitrification. Plant Cell Rep. 11:175-178.

Tsukazaki, H., M. Mii, K. Tokuhara and K. Ishikawa. 2000. Cryopreservation of *Doritaenopsis* suspension culture by vitrification. Plant Cell Rep. 19(12):1160-1164.

Van Waes, J. M. and P. C. Debergh. 1986. Adaptation of the tetrazolium method for testing the seed viability, and scanning electroscopy study of some Western European orchid. Physiol. Plant. 66:435-442.

Verleysen, H.; G. Samyn, E. Van Bockstaele and P. Debergh. 2004. Evaluation of analytical techniques to predict viability after cryopreservation. Plant Cell Tiss. Org. 77(1):11-21.

Wang, Q., M. Mawassi, N. Sahar, P. Li, V. Colova-Tsolova, R. Gafny, I. Sela, E. Tanne and A. Perl. 2004. Cryopreservation of Grapevine (*Vitis* spp.) Embryogenic Cell Suspensions by Encapsulation–Vitrification. Plant Cell Tiss. Org. 77(3):267-275.

Yamada, T., A. Sakai, T. Matsumura and S. Higuchi. 1991. Cryopreservation of apical meristems of white clover (*Trifolium repens* L.) by vitrification. Plant Sci. 73:111-116.

Yin, M. and S. Hong. 2009. Cryopreservation of *Dendrobium candidum* Wall. ex Lindl. protocorm-like bodies by encapsulation-vitrification. Plant Cell Tiss. Org. 98(2):179-185.