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Encapsulation-vitrification of *Dendrobium* sonia-28 supported by histology

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Abstract

In vitro protocorm-like bodies (PLBs) of *Dendrobium* sonia-28 were cryopreserved through an encapsulation-vitrification method. One to two and 3-4mm PLBs were precultured in half-strength semi-solid Murashige and Skoog (MS) medium supplemented with various sucrose concentrations (0, 0.25, 0.5, 0.75 and 1.0M) at different periods (0, 3, 6 and 9 days). Precultured PLBs were encapsulated and osmoprotected for 24 hours, and then dehydrated in plant vitrification solution 2 (PVS2, 0ºC) at different periods (0, 30, 60, 90, 120, 150, 180 and 210 minutes) prior to storage in liquid nitrogen (LN, -196ºC) for at least 24 hours. After rapid thawing (40±2ºC) for two minutes, the beads were unloaded with 1.2M sucrose and then cultured on half-strength semi-solid MS medium devoid of growth regulators. The 2,3,5-triphenyltetrazolium chloride (TTC) assay was used to determine the viability of the treated PLBs after two weeks of recovery. Histological analyses of non-cryopreserved and cryopreserved PLBs were conducted to assess the impact of the cryopreservation procedure on the *in vitro* PLB cultures. Observations indicated that cryopreserved PLBs underwent anatomical changes expressed as changes in the cell structure, cell wall, nucleus and cytoplasm. The optimised encapsulation-vitrification parameters involved in this study were the preculture of 3-4mm PLBs for six days in 0.5M sucrose, followed by dehydration in PVS2 at 0ºC for 150 minutes. Thus, this method was deemed promising for cryopreservation of PLBs of *Dendrobium* sonia-28.

Keywords: Cryopreservation. Encapsulation-vitrification. *Dendrobium* sonia-28. Histology.
Abrreviations: PLB Protocorm-like bodies; MS Murashige and Skoog Media; BAP Benzylaminopurine; LN Liquid Nitrogen; PVS2 Plant Vitrification Solution 2; TTC 2,3,5-Triphenytetrazolium choride.

Introduction

Orchidaceae is one of the largest families of flowering plants (Angiospermae). The genus *Dendrobium* is highly evolved and diversified, and consists of more than 1000 natural species. Cut flowers of *Dendrobium* hold the highest rank commercially due to their longevity, short production cycle and high number of flowers per inflorescence (Martin and Madassery, 2006). Alternative methods for long-term conservation of plants include cryopreservation or freeze-preservation techniques at ultra-low temperatures. Cryopreservation, defined as '*preservation in the frozen state*', is the most successful method for long-term storage of genetic resources as it theoretically stops almost all biological activity and deterioration (Lambardi et al., 2000). Encapsulation-vitrification, developed by Tannoury et al. (1991), combines the advantages of both vitrification and encapsulation–dehydration methods. Explants are encapsulated into sodium alginate beads and sufficiently dehydrated through exposure to a high osmolarity-vitrification solution, which helps induce germplasm tolerance towards ice crystal formation during exposure to liquid nitrogen (LN) (Dumet et al., 1993). Alginate, known for its polymeric inertness, easy manipulability, non-toxicity and its availability in large quantities, is the preferred encapsulation agent. The alginate capsule protects the explants during the cryopreservation procedure and reduces the chemical toxicity or osmotic stress of the vitrification solution upon the target explants (Wang et al., 2004). A viability test is able to assess the stress and cellular damages incurred in plant tissues from cryopreservation (Verleysen et al., 2004), and the 2,3,5-triphenyltetrazolium chloride (TTC) assay is a popular choice for this purpose. Histological analyses were carried out on non-cryopreserved and cryopreserved PLBs to study the anatomical changes in treated cells, and to understand the *in vitro* culture system (Yeung, 1999). In the cryopreservation of PLBs of *Dendrobium* sonia-28 by both the PVS2 (Hooi et al., 2010) and encapsulation-dehydration (Pouzi et al., 2011) methods, 3-4mm PLBs displayed higher viability compared to 1-2mm PLBs for both the control and cryopreservation treatments. The best conditions for the vitrification of the PLBs were a 24-hour preculture in 0.6M sucrose, followed by a 20-minute exposure to PVS2 at 0ºC (Hooi et al., 2010). There is great potential in investigating the use of encapsulation-vitrification in preserving endangered and commercially useful orchid germplasm. More studies must be conducted on optimising various cryopreservation parameters, such as PLB size, preculture concentration and PVS2 dehydration duration. There are a limited number of studies on the cryopreservation of orchid PLBs and there are no reports on the encapsulation-vitrification of *Dendrobium* sonia-28. This study evaluated the application of an encapsulation-vitrification protocol on PLBs of *Dendrobium* sonia-28 as a means for efficient storage of this orchid hybrid. The objectives of this study were to determine the best PLB size (1-2 or 3-4mm), preculture concentration (0, 0.25, 0.5, 0.75 and 1.0M sucrose), preculture duration (0, 3, 6 and 9 days) and PVS2 dehydration duration (0, 30, 60, 90, 120, 150, 180 and 210 minutes) in the encapsulation-vitrification of *Dendrobium* sonia-28, and to study the anatomy of non-

cryopreserved and cryopreserved PLBs of *Dendrobium* sonia-28 through histology.

Results

Optimisation of PLB size

Three to four mm PLBs displayed higher survival compared to 1-2mm PLBs in both the control and cryopreservation treatments (Fig.1). Hence, 3-4mm PLBs were used in the following treatments of this study.

Optimisation of preculture concentration

For both cryopreserved and non-cryopreserved PLBs, survival increased as the sucrose concentration increased (0-0.5M sucrose). The survival of both cryopreserved and non-cryopreserved PLBs decreased as the sucrose concentration increased from 0.75M to 1.0M sucrose. Protocorm-like bodies which were precultured in half-strength MS medium supplemented with 0.5M sucrose prior to cryostorage presented with the highest survival rate when compared to media supplemented with other sucrose concentrations (Fig. 2). Thus, the experiment was continued using 3-4mm PLBs precultured in 0.5M sucrose.

Optimisation of preculture duration

Survival increased with increasing preculture duration (0-6 days) for both cryopreserved and non-cryopreserved PLBs. The survival of both cryopreserved and non-cryopreserved PLBs then decreased as the preculture duration increased to 9 days. Protocorm-like bodies which were precultured in half-strength MS media supplemented with 0.5M sucrose for 6 days prior to cryostorage presented with the highest survival rate when compared to other preculture durations tested (Fig. 3). Thus, the experiment was continued using 3-4mm PLBs precultured in 0.5M sucrose for 6 days.

Optimisation of PVS2 dehydration duration

Early studies have indicated that all cryopreserved PLBs that were not dehydrated (0 minutes) prior to cryostorage presented with low survival rates. However, all non-cryopreserved PLBs that were not dehydrated (0 minutes) presented with high survival rates. The survival of cryopreserved PLBs increased with increasing PVS2 dehydration duration (0-150 minutes). The survival of cryopreserved PLBs then decreased when the PVS2 dehydration duration increased from 180 to 210 minutes (Fig. 4). The highest survival rate was obtained when PLBs were precultured in half-strength MS media supplemented with 0.5M sucrose for 6 days and dehydrated in PVS2 solution for 150 minutes prior to cryostorage (Fig. 4).

Histological analyses of non-cryopreserved and cryopreserved PLBs

Histological observations of non-cryopreserved and cryopreserved PLBs indicated that the majority of cells were injured either during freezing or thawing or during the osmotic dehydration step with PVS2 (Figs. 6 and 7). A small number of cells survived the cryopreservation process. Cross sections of non-cryopreserved PLBs indicated the presence of a complete outer layer of the cell wall (Fig. 5a, b), while cross sections of cryopreserved PLBs showed the breakage of this layer (Fig. 5c, d). Water that remains in cells form ice crystals that damage the cells during cryopreservation. Therefore, it is important to minimise the water content of cells in order to improve the survival rates of cryopreserved PLBs. Cross sections of non-cryopreserved PLBs indicated the presence of a dense cytoplasm, intact nuclear envelope and clear nucleoli (Fig. 6a), while cross sections of cryopreserved PLBs displayed cellular damages that indicated plasmolysis, nuclear shrinkage and ruptures in the cell wall, cellular membrane and the nuclear envelope. Most cells displayed detachment of the plasma membrane, loss of cytoplasm, slightly visible nuclei and breakage of the cell wall (Fig. 6b, c, d).

Discussion

Optimisation of PLB Size

In this study, the best result in terms of PLB size was obtained when 3-4mm PLBs were precultured in half-strength semi-solid MS medium supplemented with 0.6M sucrose, followed by 20 minutes of PVS2 dehydration at 0°C. However, alterations in the biochemical contents of cryopreserved PLBs, such as total soluble protein content, have been reported previously. The highest total peroxidase activity was reported in PLBs of *Dendrobium* sonia-28 that underwent cryostorage (Hooi et al., 2010). On the other hand, 1-2mm PLBs of *Dendrobium* sonia-28 that were subjected to the encapsulation-dehydration method produced the best viability after a 24 hour preculture in half-strength semi-solid MS medium containing 1.0M sucrose, followed by a 30-minute encapsulation process using 3.0% (w/v) sodium alginate and 0.1M $CaCl_2$. Untreated PLBs of *Dendrobium* sonia-28 produced both the highest concentration of both chlorophyll *a* and *b* in the chlorophyll test when compared to cryopreserved PLBs (Pouzi et al., 2011). Observations of photosynthetic activity in cryopreserved PLBs indicated that cryopreservation inhibited the activities of both photosystems to a certain level (Safrinah et al., 2009). Cryopreserved PLBs of *Dendrobium* sonia-28 displayed the lowest total soluble protein content when compared to untreated PLBs, which possessed the highest value of total soluble protein content (Pouzi et al*.,* 2011). Nuria and Pilar (2001) reported that this condition occurs as a plant will experience a reduction in terms of growth and metabolic activities when subjected to any biotic or abiotic stress factors. Moreover, there is a high possibility that the PLBs intended for cryopreservation possess inadequate or no stress tolerance mechanisms. In conclusion, the low protein concentrations should be interpreted as a clear sign of stress damage in plant cells (Nuria and Pilar, 2001). Protocorm like-bodies (PLBs) are the earliest unique structures that are formed during embryo development in orchid seed germination. During recovery, non-cryopreserved PLBs displayed the characteristic green colour while cryopreserved PLBs bleached or turned light yellow or brown due to osmotic shock or unfavourable regrowth conditions (Zhao et al., 2008). All cryopreserved PLBs in this research initially displayed light green colour when recovered from liquid nitrogen and incubated in the dark immediately. The PLBs bleached or turned brown after exposure to light. In the cryopreservation of shoot tips of African violet, incubating PLBs under continuous darkness can essentially reduce shock to the plant tissue (Moges et al., 2004). Protocorm-like bodies that are too small can easily suffer mechanical injury during cryopreservation. In the cryopreservation of *Ascocenda* 'Princess Mikasa' (Poobathy et al., 2009) and *Dendrobium* Bobby Messina (Antony et al., 2010), larger PLBs produced higher viability and survival

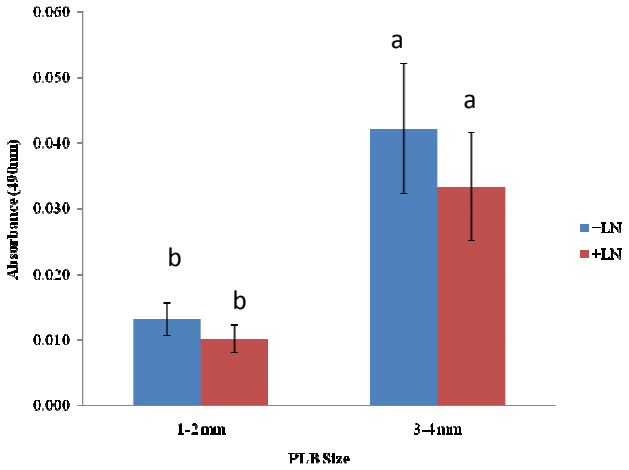

Fig 1. Effect of PLB size on viability of non-cryopreserved (-LN) and cryopreserved (+LN) PLBs of *Dendrobium* sonia-28. Bars indicate mean absorbance ± SD.

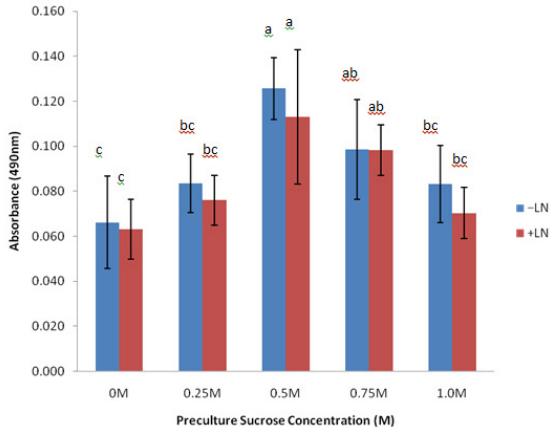

Fig 2. Effect of various sucrose preculture concentrations on the viability of non-cryopreserved (-LN) and cryopreserved (+LN) PLBs of *Dendrobium* sonia-28. Bars indicate mean absorbance ± SD.

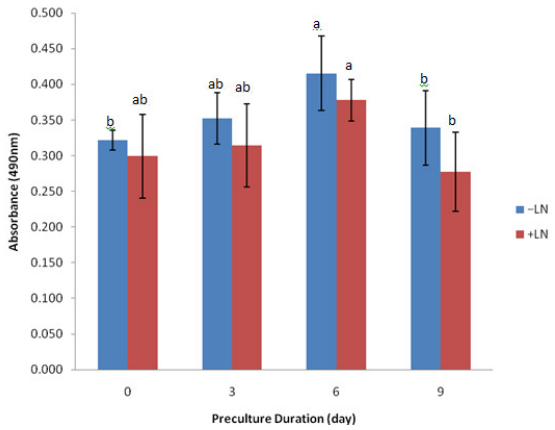

Fig 3 Effect of various preculture duration on the viability of non-cryopreserved (-LN) and cryopreserved (+LN) PLBs of *Dendrobium* sonia-28. Bars indicate mean absorbance ± SD.

rates. Therefore, the result of this study supports previous findings that larger-sized PLBs displayed higher viabilities in cryopreservation experiments.

Optimisation of preculture concentration

Sucrose is most often used as the carbon source in media. Other sugars such as glucose, maltose, mannitol and sorbitol have also been used in cryotechnology research (Sharaf et al., 2011). Generally, sucrose functions as a carbon source and as an osmotic regulator of water stress. In theory, it functions as an osmoprotectant which stabilises cellular membranes and maintains turgor in tissues (Valentovic et al., 2006; Sharaf et al., 2011). As reviewed by Sharaf et al. (2011), preculture is used to increase PLB tolerance to dehydration and subsequent freezing in liquid nitrogen (LN). High sugar concentration in the cytoplasm helps to establish a vitrified state during cryopreservation and enables cells to tolerate dehydration when preserved in LN (Yin and Hong 2009). It has been reported that the survival of cryopreserved *Medicago sativa* increased five-fold when the explants were precultured in 0.75M sucrose, compared to those precultured in 0.25M sucrose (Shibli et al., 2001). Preculture of explants in media enriched with high sucrose concentrations increases the total soluble protein and sugar contents in the treated tissues. An increase in the protein level can be considered as one of the earliest physiological responses of osmotically-stressed cells, and could be related to the improvement of the freezing tolerance of tissues (Wang et al., 2004). When *Asparagus officinalis* cells were precultured in 0.8M sucrose, the cells displayed an increase in the total soluble protein content and enhanced freezing tolerance (Jitsuyama et al., 2002). In this study, preculture in 0.5M sucrose produced the highest viability for cryopreserved and non-cryopreserved PLBs of *Dendrobium* sonia-28 PLBs (Fig. 2), and higher sucrose concentrations led to lower viability. High sucrose concentrations may cause tissue blackening and retard the proliferation of PLBs (Panis et al., 1996).

Optimisation of preculture duration

Preculture duration played an important role in the survival of cryopreserved PLBs of *Dendrobium* sonia-28. It has long been reported that preculture duration also influenced the viability and survival of cryopreserved plant tissues (Ishikawa et al., 1997; Maneerattanarungroj et al., 2007; Mohanty et al., 2012). Positive effects from extending preculture duration in high sucrose concentration were reported for *Dioscorea floribunda*, which was precultured in 0.75M sucrose for four days (Mandal and Sangeeta 2007) and for *Dendrobium* candidum, which was precultured in 0.75M sucrose for five days (Yin and Hong 2009). In this study, the viabilities of both cryopreserved and non-cryopreserved PLBs increased when they were precultured in 0.5M sucrose for six days. A three-fold increase in viability was obtained when the preculture in 0.5M sucrose was conducted for six days compared to a one-day preculture, hence its selection for subsequent stages of the experiment (Fig. 3). However, osmotolerance is rarely achieved by sucrose preculture alone. Therefore, a loading treatment with glycerol and varying amounts of sucrose is used to enhance the survival of cryopreserved plants (Hirai and Sakai 2003). A loading treatment consisting of 2M glycerol and 1M sucrose was used on PLBs of *Dendrobium candidum* to improve post-cryopreservation survival and regeneration rates. The highest survival frequency was obtained when the PLBs were loaded for 80 minutes (Yin and Hong 2009). All the findings

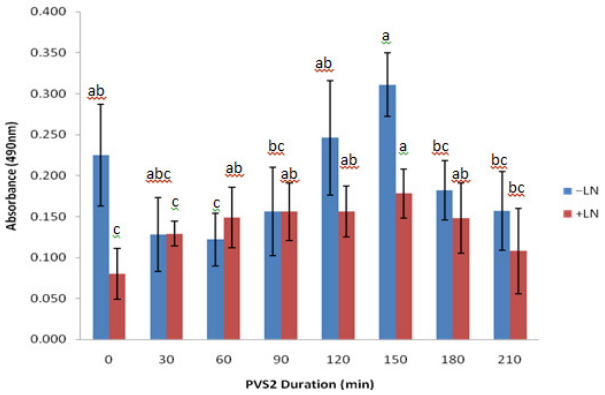

Fig 4. Effect of various PVS2 dehydration durations on the viability of non-cryopreserved (-LN) and cryopreserved (+LN) PLBs of *Dendrobium* sonia-28. Bars indicate mean absorbance ± SD.

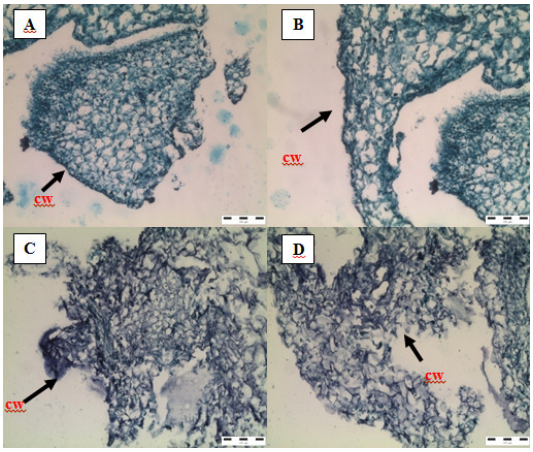

Fig 5. Histological section of *Dendrobium* sonia-28. (A and B) Cross sections of untreated or non-cryopreserved PLBs; (C and D) cross sections of cryopreserved PLBs (Scale bar = 200µm) [cw= cell wall].

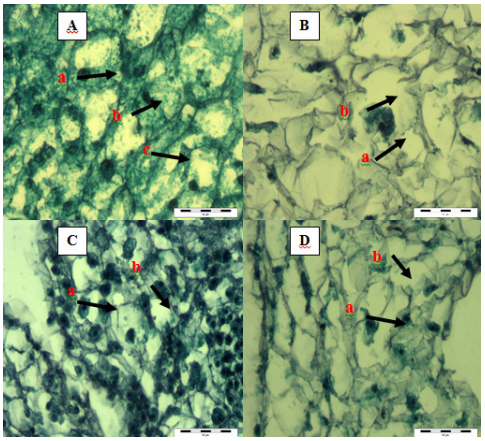

Fig 6. Histological sections of *Dendrobium* sonia-28. (A) Cross section of untreated or non-cryopreserved PLBs; (B, C and D) cross sections of cryopreserved PLBs (Scale bar = 50µm) [a= nucleus, b= cell wall, c= cytoplasm].

suggested that different explants or species exhibited varying levels of tolerance to high sucrose concentrations.

Optimisation of PVS2 dehydration duration

In any encapsulation-vitrification protocol, precultured explants should be sufficiently dehydrated by exposing them to a highly-concentrated vitrification solution such as PVS2, to protect the tissues against desiccation injuries and to avoid the formation of ice crystals during immersion in LN (-196ºC). However, over-exposure of tissues to PVS2 may cause chemical toxicity and excessive osmotic stress (Wang and Perl 2006; Fabian et al., 2008; Suranthran et al., 2012). There are two important factors that affect the survival of cryopreserved plant tissues: explant dehydration period and temperature of the vitrification solution (Sen-Rong and Ming-Hua 2009). The optimal PVS2 exposure time varies with species and depends on the temperature of the solution during the treatment. Many reports have shown that dehydration at 0ºC reduces the toxicity of the PVS2 and usually yields higher explant survival rates, when compared to exposure at room temperature. The PVS2 incubation time can also be extended at 0ºC, thus allowing flexibility when handling a large number of samples at the same time (Yin and Hong 2009). The survival frequency of cryopreserved *Dendrobium candidum* PLBs treated with PVS2 increased from 76.2% when dehydrated at 25ºC for 120 minutes to 89.4% when dehydrated at 0ºC for 150 minutes (Yin and Hong 2009). In this study, the highest survival rate of cryopreserved PLBs was obtained when they were dehydrated in PVS2 at 0ºC for 150 minutes (Fig. 4).

Histology analyses of untreated and cryopreserved PLBs of Dendrobium sonia-28

In this study, histological analyses were conducted to investigate the tissue damages that occurred during the explants' dehydration and freezing (Figs. 5, 6). Cryopreservation procedures may result in cell distortion, cell and nuclear shrinkage, nuclear envelope rupture and plasmolysis (Wen et al., 2010). As reviewed by Koichi et al. (2012), the primary site of freezing injury is reflected to be the plasma membrane and such cryoprotective effects might vary with the composition of the plasma membrane. Similar results were obtained in this study. The cell wall and the plasma membrane were the sites of cellular injury during cryopreservation. Following the freeze-thaw cycles, the membrane permeability, corresponding to cellular viability, has frequently decreased (Fujikawa 1995). The damages and ultrastructural changes in the cells could be attributed to intracellular or extracellular ice formation. The major factor of cryopreservation failure was linked to lethal intracellular ice crystal formation (Mazur 1984; Wen et al., 2010).

Materials and methods

Plant material

In this study, *in vitro* cultures of protocorm-like bodies (PLBs) of *Dendrobium* sonia-28 were selected for cryopreservation. The PLB stock culture was cultured on half-strength semi-solid Murashige and Skoog (1962) medium supplemented with 1mg.L^{-1} 6-benzylaminopurine (BAP), 20g.L^{-1} sucrose and 2.75g.L^{-1} gelrite. The cultures were incubated at 25±2ºC under 16 hours photoperiod using cool white fluorescent lamps (Philips TLD, 36W,

150µmol.m^{-2}.s^{-1}). The resulting PLB clumps were then subcultured for every 4 weeks.

Preculture

For the optimisation of PLB size, 1-2 and 3-4 mm PLBs were selected from four-week old cultures and precultured in half-strength semi-solid MS media supplemented with 0.5M sucrose at 25°C for 24 hours under 16 hours photoperiod. For the optimisation of sucrose preculture concentration, PLBs selected from four-week old culture were precultured in half-strength semi-solid MS media supplemented with different concentrations of sucrose (0, 0.25, 0.5, 0.75 and 1M) at 25°C for 24 hours under 16 hours photoperiod. For the optimisation of preculture duration, PLBs selected from four-week old culture were precultured in half-strength semi-solid MS medium supplemented with 0.5M sucrose for 0, 3, 6 and 9 days under 16 hours photoperiod.

Encapsulation, osmoprotection, dehydration

The PLBs were individually encapsulated in alginate beads (about 4-5 mm in diameter) by transferring them from liquid medium containing 3% sodium alginate into liquid 0.1M calcium chloride medium, both supplemented with 0.4M sucrose, 1M glycerol and half-strength liquid MS components. After 30 minutes of gentle stirring, the beads were collected and osmoprotected in half-strength liquid MS medium supplemented with 0.75M sucrose and 1M glycerol on an orbital shaker (110 rpm) at 24°C for 24 hours under 16 hours photoperiod. The beads were then dehydrated in PVS2 at the following durations: 0, 30, 60, 90, 120, 150, 180 and 210 minutes.

Cryostorage, thawing and recovery

The beads were surface-dried by blotting them on sterile filter paper. They were then placed in sterile cryovials and directly plunged into LN for 24 hours. Cryovials removed from LN were rapidly thawed in a water bath set at 40±2ºC for two minutes. The beads were washed with liquid half-strength MS medium supplemented with 1.2M sucrose for 20 minutes and then surface-dried by blotting them on sterile filter paper. The beads were then cultured on half-strength semi-solid MS medium supplemented with 2% sucrose and devoid of growth regulators for three weeks: the beads were placed in the dark for a week, followed by placement in dim light (3.4µmol.m^{-2}.s^{-1}) for another week and under 16 hours photoperiod using cool white fluorescent lamps (Philips TLD, 36 W, 150µmol.m^{-2}.s^{-1}) in the third week.

Survival assessment

After three weeks of recovery, the survival of non-cryopreserved and cryopreserved PLBs were assessed based on growth observations and viability assay via the 2,3,5-triphenyltetrazolium chloride (TTC) spectrophotometry analysis at 490nm (Verleysen et al., 2004).

Histological analysis

Histological analyses were performed for non-cryopreserved and cryopreserved PLBs. The preparation of histological slides was conducted through the following steps. First, the PLBs were transferred into the TBA alcohol series at various concentrations (50-100%). The PLBs were then treated with TBA I, TBA II and TBA III and left overnight. The PLBs were exposed to xylene, Wax I, II, and III. The specimen was blocked and sliced using a microtome set at six microns (Leica RM 2135). The specimen was then stained with safranin and fast green. The slides were observed using a light microscope (Olympus BX41).

Statistical Analysis

The treatments consisted of six replicates containing 10 PLBs per replicate. Means were compared through the independent samples t-test in the selection of the best PLB size for vitrification. Means obtained from the remaining treatments were analysed with one-way ANOVA and differentiated with Tukey's test. The probability value for both the tests was set at 0.05, using the Statistical Package for Social Sciences (SPSS), version 16.0.

Conclusion

The optimised encapsulation-vitrification protocol for PLBs of *Dendrobium* sonia-28 involved the preculture of 3-4mm PLBs in 0.5M sucrose for six days, followed by encapsulation, osmoprotection and dehydration in PVS2 for 150 minutes at 0ºC prior to freezing. Further optimisation of new parameters in this protocol could ensure the production of higher survival and regeneration rates of PLBs of *Dendrobium* sonia-28, and also facilitate the application of this protocol to a broader range of plant and orchid species. Histological analyses indicated that the damages from the cryopreservation procedure resulted from the formation of ice crystals in the PLBs.

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References

Antony JJJ, Keng CL, Rathinam X, Sinniah UR, Subramaniam S (2010) Preliminary study on cryopreservation of *Dendrobium* Bobby Messina protocorm-like bodies by vitrification. Afr J Biotechnol 9(42): 7063-7070

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

Fabian A, Jager K, Darko E, Barnabas B (2008) Cryopreservation of wheat (*Triticum aestivum* L.) egg cells by vitrification. Acta Physiol Plant 30:737–744

Fujikawa S (1995) A Freeze-fracture study designed to clarify the mechanisms of freezing injury due to the freezing-induced close apposition of membranes in cortical parenchyma cells of mulberry. Cryo Bio 32: 444-454

Hirai D, Sakai A (2003) Simplified cryopreservation of sweet potato (*Ipomoea batatas* L. by optimizing conditions for osmoprotection. Plant Cell Rep 21: 961–966

Hooi TH, James J, Julkiflee A, Poobathy R, Gnasekaran P, Subramaniam S (2010) A Novel Approach for Preliminary PVS2 Vitrification Optimisation Parameters of *Dendrobium Sonia*-28 Orchid with Evans Blue Staining. Adv Environ Biol 4(2): 284-290

Ishikawa K, Harata K, Mii M, Sakai A, Yoshimatsu K, Shimomura K (1997) Cryopreservation of zygotic embryos

of a Japanese terrestrial orchid (*Bletilla striata*) by vitrification. Plant Cell Rep 16:754–757

Jitsuyama Y, Suzuki T, Harada T, Fujikawa S (2002) Sucrose incubation increases freezing tolerance of asparagus (*Asparagus officinalis* L.) embryogenic cell suspensions. Cryo-Letters 23:103-112

Koichi N, Keiji D, Kazuyoshi K (2012) Cryopreservation of four valuable strains of microalgae, including viability and characteristics during 15 years of cryostorage. J Appl Phycol In Press

Lambardi M, Fabbri A, Caccavale A (2000) Cryopreservation of white poplar (*Populus alba* L.) by vitrification of *in vitro*-grown shoot tips. Plant Cell Rep 19: 213-218

Mandal BB, Sangeeta AG (2007) Regeneration of *Dioscorea floribunda* plants from cryopreserved encapsulated shoot tips: effect of plant growth regulators. Cryo-Letters 28: 329-336

Maneerattanarungroj P, Bunnag S, Monthatong M (2007) *In vitro* conservation of *Cleisostoma areitinum* (Rchb.f.) Garay, rare Thai orchid species by an encapsulation-dehydration method. Asian J Plant Sci 6:1235–1240

Mohanty P, Das MC, Kumaria S, Tandon P (2012) High-efficiency cryopreservation of the medicinal orchid *Dendrobium nobile* Lindl. Plant Cell Tissue Org Cult 109 (2): 297-305.

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

Mazur P (1984) Freezing of living cells: mechanisms and applications. Am J Physiol 247: 125–142

Moges A, Shibli R, Karam N (2004) Cryopreservation of African violet (*Saintpaulia ionantha* Wendl.) shoot tips. In Vitro Cell Dev – P 40: 389-395

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

Nuria PB, Pilar RT (2001) Protein Content Quantification By Bradford Method. In: Handbook of Plant Ecophysiology Techniques. Kluwer Academic Publisher, pp. 283-295

Panis B, Totté N, Van Nimmen K, Withers LA, Swennen R (1996) Cryopreservation of banana (*Musa* spp.) meristem cultures after preculture on sucrose. Plant Sci 95-106

Poobathy R, Nair H, Subramaniam S (2009) Optimisation of encapsulation-dehydration protocol for the orchid hybrid *Ascocenda* 'princess mikasa'. Adv Environ Biol 3: 69-83

Pouzi NZ, Rathinam X, Antony JJJ, Poobathy R, Subramaniam S (2011) Early investigation on cryopreservation of *Dendrobium* sonia-28 using encapsulation-dehydration with modified Evans blue assay. Afr J Biotechnol 10(18): 3534-3539

Safrinah R, Xavier R, Uma Rani S, Sreeramanan S (2009) Optimisation of Cryopreservation Technique in *Mokara* golden nugget orchid Using PVS2 Vitrification. Int J Agr Res 4: 218-227

Sen-Rong H, Ming-Hua Y (2009) High-efficiency vitrification protocols for cryopreservation of *in vitro* grown shoot tips of rare and endangered plant. Plant Cell Tissue Org Cult 99: 217-226

Sharaf SA, RA Shibli, MA Kasrawi, SH Baghdadi (2011) Cryopreservation of wild Shih (*Artemisia herba-alba* Asso.) shoot-tips by encapsulation-dehydration and encapsulation-vitrification. Plant Cell Tissue Org Cult In Press

Shibli RA, Haagenson DM, Cunningham SM, Berg WK, Volenec JJ (2001) Cryopreservation of alfalfa (*Medicago sativa* L.) cells by encapsulation–dehydration. Plant Cell Rep 20: 445–450

Suranthran P, Gantait S, Sinniah UR, Subramaniam S, Alwee SSRS, SH Roowi (2012) Effect of loading and vitrification solutions on survival of cryopreserved oil palm polyembryoids. Plant Growth Regul 66: 101-109

Tannoury M, Ralambosoa J, Kaminski M, Dereuddre J (1991) Cryopreservation by vitrification of coated tips of carnation (*Dianthus caryophyllus L.*) cultured *in vitro*. Comptes Rendus de l' Acad. des Science Paris Serie III 313: 633-638

Valentovic P, Luxova M, Kolarovic L, Gasparikova O (2006) Effect of osmotic stress on compatible solutes content, membrane stability and water relations in two maize cultivars. Plant Soil Environ 52(4): 186-191

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

Wang Q, Mawassi M, Sahar N, Li P, Violeta CT, Gafny R, Sela I, Tanne E, Perl A (2004) Cryopreservation of grapevine (*Vitis* spp.) embryogenic cell suspensions by encapsulation–vitrification. Plant Cell Tissue Org Cult 77: 267-275

Wang Q, Perl A (2006) Cryopreservation of embryogenic cell suspensions by encapsulation-vitrification. Methods Mol Biol 318: 77–86

Wen B, Wang R, Cheng H, Song S (2010) Cytological and physiological changes in orthodox maize embryos during cryopreservation. Protoplasma 239: 57-67

Yeung E (1999) The use of histology in the study of plant tissue culture systems—Some practical comments. In Vitro Cell Dev Pl 35: 137-143

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

Zhao P, Wu F, Feng FS, Wang WJ (2008). Protocorm-like body (PLB) formation and plant regeneration from the callus culture of *Dendrobium candidum* Wall *ex* Lindl. In Vitro Cell Dev Pl 44: 178-185