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Molecular stability of protocorm-like bodies of *Dendrobium* sonia-28 after encapsulation-dehydration and vitrification

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Abstract

Dendrobium sonia-28 is prized for its pink-coloured and good cut flowers. Cryopreservation, requiring little space and maintenance, is touted as an important tool for long-term storage of plant genetic resources. The vitrification and encapsulation-dehydration methods of cryopreservation were applied on protocorm-like bodies (PLBs) of the orchid hybrid *Dendrobium* sonia-28, with survival and stability assessments conducted through growth observations and RAPD analysis respectively. Results obtained from both the control encapsulation-dehydration (53.3%) and vitrification (30.0%) experiments indicated that the encapsulation-dehydration treatment is less damaging to non-cryopreserved PLBs. However, the vitrification treatment produced PLBs that survived cryopreservation (16.0%). No surviving PLBs were obtained from the encapsulation-dehydration experiment. The RAPD analyses of PLBs of *Dendrobium* sonia-28 indicated that PLBs that were vitrified and cryopreserved were genetically stable, while those that were encapsulated, dehydrated and then cryopreserved were not genetically stable when compared to the stock culture.

Keywords: Cryopreservation; *Dendrobium* sonia-28; Encapsulation-dehydration; Protocorm-like bodies; RAPD; Vitrification.
Abbreviations: DMSO, dimethyl sulfoxide; LN, liquid nitrogen; MS, Murashige and Skoog medium; PLBs, protocorm-like bodies; PVS2, plant vitrification solution 2; RAPD, rapid amplification of polymorphic DNA; rpm, revolutions per minute.

Introduction

Orchids of the genus *Dendrobium* are epiphytes possessing connected stems known as pseudobulbs, each with the capacity of producing one or few inflorescence (Yue et al., 2006). International production of potted *Dendrobium* plants spiked up in the last few years, with large-scale production occurring in the Netherlands, Germany, China, Taiwan, Thailand, Philippines, Unites States, and Japan (Puchooa, 2004). *Dendrobium* sonia-28, a hybrid resulting from the cross between two hybrids, *Dendrobium* Caesar and *Dendrobium* Tomie Drake, is prized for its pink-coloured and good cut flowers. Categorised as a *Dendrobium Phalaenopsis* orchid, the hybrid are popular as pot plants due to their long-lasting flower sprays (Van Rooyen Orchids Catalogue, 2007). Problems associated with the genus include low or no seed setting and germination, and heterozygous seedling progenies that are not true-to-type plants of hybrid cultivars (Martin and Madassery, 2006). Cryopreservation, requiring little space and maintenance, is touted as an important tool for long-term storage of plant genetic resources (Sakai et al., 2008). The balance between the exit of intracellular water, ice formation and cell solute concentration is important for successful cryopreservation (Benson, 2008). *In vitro* cryopreservation is currently widely experimented on members of the Orchidaceae as the technique is not readily applicable on orchids due to specific features of these plants (Pritchard and Seaton, 1993; Popov et al., 2004; Bukhov et al., 2006). Plants can be cryopreserved through a method termed as vitrification, which is defined as the process in which liquid turns to solid without undergoing crystallisation (Fahy et al., 1984; Engelmann et al., 2008). The vitrification method capitalise on the exposure of cells and tissues to highly concentrated (7-8 M) cryoprotectants, allowing direct immersion in LN and circumventing the freeze-concentration step (Sakai et al., 2008). The new vitrification method also includes a technique termed as encapsulation-dehydration, which was first used to cryopreserve pear and potato (*Solanum phureja)* shoot tips (Dereuddre et al., 1990; Fabre and Dereuddre, 1990; Engelmann et al., 2008). The encapsulation step protects the explant while allowing exposure to extreme conditions that are usually deleterious to unencapsulated explants, for instance pretreatment in high sucrose concentrations and dehydration to low moisture contents. The dehydration process removes most or all water from the encapsulated explants, allowing vitrification of the intercellular solutes as an explant is exposed to LN and avoiding lethal intracellular ice crystallization (Engelmann, 1997; Engelmann et al., 2008). Theoretically, cryopreservation prevents the occurrence of genetic change in the target cells that could possibly be induced through *in vitro* culture selection. However, current evidence from studies of other cryopreserved organisms and germplasm indicate that ultra-low storage temperatures are not as stable as was once thought (Walters et al., 2004; Benson et al., 2007). Most assessments of genetic stability in organisms recovered and regenerated from the cryopreserved state are conducted at the phenotypic, histological, cytological, biochemical and molecular levels (Harding, 1999). The random amplification of polymorphic DNA (RAPD)

technique is a popular method for estimating genetic diversity in various plant populations as it presents with several advantages such as speedy results, low cost and the requirement of small sample amounts (Huff et al., 1993; Ge et al., 1999; Nybom and Bartish, 2000; Kingston et al., 2004; Li and Ge, 2006). The RAPD markers have been used to classify various orchid genera such as *Phalaenopsis* (Goh et al., 2005; Chen et al., 1994; Khosravi et al., 2009), *Goodyera* (Wong and Sun, 1999; Khosravi et al., 2009) and *Zeucine* (Sun and Wong, 2001; Khosravi et al., 2009). The objective of this research was to assess the genetic stability of protocorm-like body (PLB) samples of *Dendrobium* sonia-28 that were subjected to the encapsulation-dehydration and vitrification cryopreservation techniques, using the RAPD technique.

Results

Differences in PLB survival rates between encapsulation-dehydration and vitrification treatments

Results obtained from both the control encapsulation-dehydration and vitrification experiments indicated that the encapsulation-dehydration treatment is less damaging to the non-cryopreserved PLBs when compared to the vitrification treatment (Table 1). Growth in PLBs subjected to the control treatments occurred in two ways: from their previous stage of growth prior to the treatment or through the proliferation of new PLBs on the mother PLB (Figs. 1a and 1b). The control PLBs bleached, but produced new PLB clumps within three weeks of growth recovery. Cryopreserved PLBs bleached within three days of exposure to 16/8 photoperiod despite showing initial growth and proliferation. No growth was observed for cryopreserved PLBs that were subjected to the encapsulation-dehydration treatment, even after three months of recovery (Fig. 1c). This indicated that the encapsulation-dehydration method may need to be further optimised for successful cryopreservation of this orchid. In the vitrification treatment, no visible growth was observed in cryopreserved PLBs in the initial weeks of the growth recovery period. However, new PLB growth was observed on cryopreserved PLBs beyond the fifth week of recovery (Fig. 1d). This could be the effect of preservation of single cells or clumps of cells within the PLB, rather than the entire organ. Successfully cryopreserved cells could have taken time to grow and expand, with growth only visible to the naked eye from the fifth week onwards. The growth of the cryopreserved PLBs then resumed, although slow, as that observed in untreated PLBs, beginning from the sixth week of recovery.

DNA amplification from untreated, non-cryopreserved and cryopreserved PLB samples of Dendrobium sonia-28

Out of the 15 RAPD primers used for all five types of samples, nine primers produced amplified DNA fragments from untreated control PLB samples. A total of 11 primers produced amplifications in DNA samples from vitrified and non-cryopreserved PLBs. Twelve primers amplified DNA segments in both vitrification-cryopreserved PLBs and non-cryopreserved encapsulated and dehydrated PLBs. Four primers produced amplifications in DNA samples from cryopreserved encapsulated and dehydrated PLBs. The 15 primers yielded a total of 82 bands in the untreated control PLB sample, 91 bands for the vitrification-control PLB sample, 101 bands in the vitrified PLB sample, 98 bands for the non-cryopreserved encapsulated and dehydrated PLB sample, and 14 bands for the cryopreserved encapsulated and dehydrated PLB sample, yielding a grand total of 386 bands in the entire RAPD experiment. The sizes of the DNA fragments generated from the samples varied from 200 bp to 2000 bp. The number of amplified DNA fragments produced from each compatible primer ranged from one to 13 bands (Fig. 2). Among the 91 DNA bands produced in samples obtained from the vitrification-control PLBs, 72 bands or 79.1% were monomorphic to those produced in the untreated control plant, while 19 bands were polymorphic (Supplementary Table 1). Eight primers scored an SI value of 1.0 (Table 2). Among the 101 DNA bands produced in samples obtained from the vitrified PLBs, 82 bands or 81.2% were monomorphic to those produced in the untreated control plant, while 19 bands were polymorphic (Supplementary Table 2). All nine primers that produced DNA amplification in the control untreated PLB sample also produced monomorphic bands in DNA samples obtained from vitrified PLBs, with all nine primers used scoring an SI value of 1.0 (Table 2). Among the 98 DNA bands produced in samples obtained from encapsulated, dehydrated and non-cryopreserved PLBs, 79 bands or 80.6% were monomorphic to those produced in the untreated control plant, while 19 bands were polymorphic (Supplementary Table 3). A total of eight primers scored an SI value of 1, while the rest scored 0.0 or could not be calculated, with the exception of the primer OPB18, which scored 0.4 (Table 2). All 14 bands generated from samples obtained from encapsulated, dehydrated and cryopreserved PLBs were polymorphic with respect to the control sample (Supplementary Table 4). Nine out of 15 amplified DNA samples from encapsulated, dehydrated and cryopreserved PLBs scored an SI of 0. The SI values of the rest of the samples could not be calculated. The RAPD analyses of all five samples suggest that there is no genomic variation in three of the treated samples: vitrification-control PLBs, vitrified PLBs and encapsulated, dehydrated and non-cryopreserved PLBs. Genetic variation was observed in the DNA samples of PLBs that were encapsulated, dehydrated and cryopreserved.

Discussion

Differences in PLB survival rates between encapsulation-dehydration and vitrification treatments

It is important to maintain temperatures of cryopreserved materials during sample withdrawal from the bank, to consider precryogenic storage handling and the sample's physiological health status and to correctly choose the cryoprotectant and storage regime (liquid- or vapour-phase nitrogen) as the information affects the stability of glasses formed, which in turn is primarily affected by both the choice of cryoprotectant and its mode of action (Benson et al., 2007). For instance, from the thermal analysis of PVS2, it was found that the cryoprotectant was particularly efficient as it is able to restrict molecular mobility and disorganize ice crystal structure in samples (Volk and Walters, 2006; Benson et al., 2007). Based on this premise, the encapsulation-dehydration method could have been an unsuitable cryopreservation method for the PLBs of *Dendrobium* sonia-28, as the method could not confer adequate protection for PLBs intended for storage in liquid nitrogen. Direct immersion of tissues in LN usually requires a range of cryoprotective treatments, and the most common approach involves the treatment of samples with PVS2 for 30 to 90 minutes. Toxicity could occur to the samples due to extended incubations in the cryoprotectant (Lambardi et al., 2008). This was evident in the case of non-cryopreserved PLBs of

Table 1. Survival percentages of PLBs of *Dendrobium* sonia-28 subjected to encapsulation-dehydration or vitrification treatments, after three months of post-cryopreservation recovery.

Treatment	Survival percentage	
	-LN	+LN
Encapsulation-dehydration	53.3a	0c
Vitrification	30.0b	16.0bc

Means with the same letters are not significantly different.

Fig 1.(a) Non-cryopreserved PLBs subjected to encapsulation-dehydration immediately resumed growth or proliferated a few days after the recovery treatment; b) non-cryopreserved PLBs subjected to encapsulation-dehydration resumed growth from their previous state prior to the entire encapsulation process and produced new PLBs upon surfaces of bleached or browning tissues; c) cryopreserved PLBs subjected to encapsulation-dehydration bleached at the growth recovery stage; d) regrowth of cryopreserved PLBs in the vitrification experiment was only observed as the development of new PLBs from the original PLBs that were undergoing browning or hyperhydricity. Bar = 2 mm.

Dendrobium sonia-28 that were subjected to vitrification, which produced lower recovery rates when compared to PLBs subjected to the encapsulation-dehydration protocol. 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 0.4 M 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 (Tsukazaki et al., 2000). Hence, in the case of *Dendrobium* sonia-28, a compromise was required between the PVS2 toxicity and immersion time for successful cryopreservation of the PLBs. Recovery rates may also be adversely affected by photooxidative stress and free radicals (Touchell and Walters, 2000; Normah and Makeen, 2008). High recovery rates of cryopreserved explants can be sustained when the recovery media are designed to suppress production of free radicals or provide free radical scavenging elements (Touchell and Walters, 2000; Normah and Makeen, 2008). The supplementation of ascorbic acid and charcoal to the recovery media of vitrification-treated PLBs of *Dendrobium* sonia-28 could have been the reason behind the success of the cryopreservation exercise, when compared to the encapsulation-dehydration protocol.

RAPD analysis of untreated, encapsulated-dehydrated and vitrified PLBs of Dendrobium sonia-28

The RAPD analyses of stock PLBs, and control and cryopreserved PLBs of *Dendrobium* sonia-28 suggest that there is no genomic variation in three of the treated samples: vitrification-control PLBs, vitrified PLBs and encapsulated, dehydrated and non-cryopreserved PLBs. Genomic variation may have occurred in PLBs that were encapsulated, dehydrated and cryopreserved, as polymorphic band formation was observed when DNA samples of the PLBs were amplified with some primers, while some primers were not able to produce DNA amplifications at all. The genomic differences could be attributed to the cryostorage step itself, as non-cryopreserved encapsulated-dehydrated PLBs did not differ genetically from the stock plantlets, and were morphologically and phenotypically similar to the stock plantlets as well. This study showed that cryopreserved PLBs of *Dendrobium* sonia-28 that were subjected to vitrification were genetically stable throughout the entire procedure, indicating that the vitrification method is safe to be used for the preservation of this orchid. Although not much knowledge has been generated on the topic of post-cryopreservation genetic stability in various organisms, outcomes of genetic stability assessments in over 100 cryopreserved *in vitro* higher plants samples over the past 25 years indicates that storage in LN maintains genetic stability (Benson et al., 2007). It has been long suspected that dimethyl sulfoxide (DMSO), used in many cryopreservation protocols, causes genetic changes in animal, protist and plant cells (Ipser, 1992; Vannini and Poli, 1983; Martín and González-Benito, 2005). However, no genomic variations were detected in the regenerated material from most cryopreservation experiments, with many variations occurring as a result of the *in vitro* proliferation or regeneration process rather than the cryopreservation procedure itself (Harding, 1997; Martín and González-Benito, 2005). No modifications were detected at the morphological, agronomical, chromosomal, biochemical and/or molecular levels for the cryopreserved explants of sugarcane (Gonzalez-Arnao, 1996), apple (Hao et al., 2002), yam (Sangeeta et al., 2002; Sonali-Dixit et al., 2005), kiwi and grape (Zhai et al., 2003) when the tests were conducted after the thawing step (Engelmann et al., 2008). Variation was also discovered in one regenerated explant of *Dioscorea* that was initially subjected to encapsulation and dehydration prior to cryostorage, although a comparison of the phenomenon was not conducted with other cryopreservation

Table 2. Similarity indices from RAPD analyses of DNA samples obtained from PLBs of *Dendrobium* sonia-28 subjected to the vitrification and encapsulation-dehydration experiments.

Primer	Similarity index for control vitrification experiment	Similarity index for vitrified and cryopreserved PLBs	Similarity index for control encapsulation-dehydration experiment	Similarity index for encapsulated, dehydrated and cryopreserved PLBs
OPA04	1.0	1.0	1.0	0.0
OPAW13	1.0	1.0	1.0	0.0
OPB02	1.0	1.0	1.0	0.0
OPB11	1.0	1.0	1.0	0.0
OPB12	1.0	1.0	1.0	0.0
OPB17	1.0	1.0	1.0	0.0
OPB18	1.0	1.0	0.4	0.0
OPG14	0.0	1.0	1.0	0.0
OPG15	1.0	1.0	1.0	0.0
OPAW17	0.0	0.0	0.0	-
OPB05	-	-	-	-
OPG03	0.0	0.0	0.0	-
OPG13	0.0	0.0	0.0	-
OPB13	-	-	-	-
OPD01	-	-	-	-

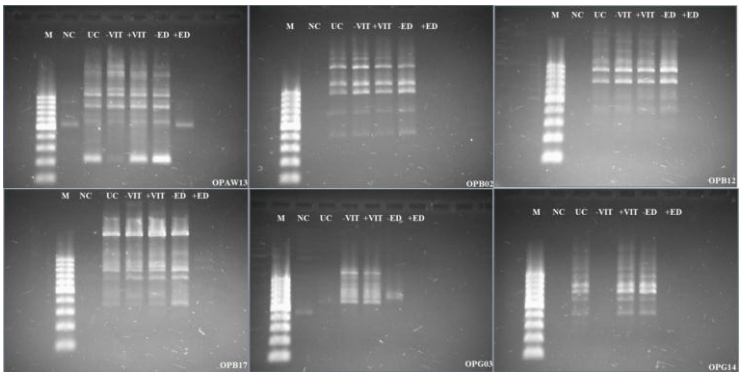

Fig 2. RAPD results for DNA samples obtained from untreated control PLBs (UC), vitrification-control PLBs (-VIT), vitrified PLBs (+VIT), encapsulated, dehydrated and non-cryopreserved PLBs (-ED) and encapsulated, dehydrated and cryopreserved PLBs (+ED), using the primers (clockwise from top left) OPAW13, OPB02, OPB12, OPG14, OPG03 and OPB17. M = 100 bp DNA ladder, NC = negative control consisting of all PCR components except for the PLB DNA sample.

methods (Dixit et al., 2003; Martín and González-Benito, 2005). Polymorphism was also detected between both cryopreserved and non-cryopreserved slow-frozen and encapsulated and dehydrated *Prunus* plantlets, detected through the use of AFLP. However, all regenerated plantlets were phenotypically true-to-type (Helliot et al., 2002; Martín and González-Benito, 2005). RAPD assays conducted for embryogenic tissues of *Pinus nigra* Am. cryopreserved through a slow-freezing method (Salaj et al., 2011) and encapsulated microshoots of Picrorhiza kurrooa (Mishra et al., 2011) confirmed the genetic fidelity of the treated tissues. No differences were detected in the relative DNA content of control and cryopreserved shoot tips of *Rabdosia rubescens* when the tissues were subjected to a flow cytometry analysis after an encapsulation-dehydration procedure. However, a sequence-related amplified polymorphism (SRAP) assay revealed that one cryopreserved line displayed variation in the banding pattern, necessitating the monitoring of the molecular stability of the recovered shoots (Ai et al., 2012).

Materials and methods

Propagation of plant materials and preparation of experimental media

Protocorm-like body cultures of *Dendrobium* sonia-28 were initiated by aseptically culturing seeds of the hybrid in half-strength semi-solid MS medium supplemented with 2% sucrose, 2.75 gL^{-1} Gelrite™ and 1 $mgmL^{-1}$ 6-benzylaminopurine (BAP). The resulting cultures were chopped into clumps of two to three PLBs and subcultured every four weeks. The cultures were incubated at 25 ± 2ºC under 16 hours photoperiod using cool white fluorescent lamps (Philips TLD, 36 W, 150 $\mu molm^{-2}s^{-1}$). All media used in the vitrification and encapsulation-dehydration experiments were supplemented with half-strength MS (Murashige and Skoog, 1962) medium components. Media used in the vitrification experiment were also supplemented with 0.6 µM L-ascorbic acid. The stock ascorbic acid was filter-sterilized using a 0.45 µm syringe filter and a 10 mL syringe prior to addition in autoclave-sterilized media. The semi-solid preculture medium was supplemented with 2.75 gL^{-1} Gelrite™ and 0.4 M sucrose, and poured into sterile plastic Petri dishes. The loading solution contained 0.4 M sucrose and 2.0 M glycerol. The plant vitrification solution 2 (PVS2) consisted of 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) DMSO and 0.4 M sucrose (Sakai et al., 1990), and the unloading solution contained 1.2 M sucrose. The semi-solid recovery medium was supplemented with 2% sucrose, 2 gL^{-1} charcoal and 2.75 gL^{-1} Gelrite™. In the encapsulation-dehydration experiment, the liquid preculture medium was prepared in 125 mL Erlenmeyer flasks, 25 mL to a flask, and supplemented with 0.25 M, 0.50 M or 0.75 M sucrose. The sodium alginate medium was supplemented

Table 3. Primers used in the amplification of DNA segments obtained from cryopreserved and non-cryopreserved PLB samples of *Dendrobium* sonia-28

Primers	Sequence (5'-3')	G+C Content (%)	T_m (°C)
OPA04	AATCGGGCTG	60	39.5
OPAW13	CTACGATGCC	60	39.5
OPAW17	TGCTGCTGCC	70	43.6
OPB02	TGATCCCTGG	60	39.5
OPB05	TGCGCCCTTC	70	43.6
OPB11	GTAGACCCGT	60	39.5
OPB12	CCTTGACGCA	60	39.5
OPB13	TCCCCCCGCT	70	43.6
OPB17	AGGGAACGAG	60	39.5
OPB18	CCACAGCAGT	60	39.5
OPD01	ACCGCGAGGG	70	43.6
OPG03	GAGCCCTCCA	70	43.6
OPG13	CTCTCCGCCA	70	43.6
OPG14	GGATGAGACC	70	43.6
OPG15	ACTGGGACTC	60	39.5

with 3.0% (w/v) sodium alginate and 0.2 M sucrose. The encapsulation agent was supplemented with 0.1 M calcium chloride dihydrate and 0.2 M sucrose. The liquid recovery medium was prepared in 25 mL batches in 125 mL Erlenmeyer flasks, and consisted of half-strength MS components, supplemented with 2% sucrose. The pH of all media was adjusted to 5.8 prior to autoclaving.

Vitrification procedure for PLBs of Dendrobium sonia-28

In this experiment, 3-4 mm friable PLBs of *Dendrobium* sonia-28 were selected from four week-old cultures using a 2 mm × 2 mm gridlock graph paper, and precultured in semi-solid half-strength MS medium supplemented with 0.4 M sucrose for 48 hours. The PLBs were then immersed in 1.5 mL of loading solution for 20 minutes and dehydrated in PVS2 at 0°C for 50 minutes in 2 mL cryovials (Nalgene Nunc, USA) before direct plunging into liquid nitrogen (-196°C) for 24 hours (MVE LAB 20). Frozen PLBs were thawed in a 40 ± 2°C water bath for 90 seconds. Storage in LN and thawing were omitted for non-cryopreserved PLBs. The PVS2 solution was immediately removed from the cryovials. Both cryopreserved and non-cryopreserved PLBs were then immersed in 1.5 mL unloading solution for 20 minutes at room temperature, with an exchange of fresh solution at 10 minutes intervals. The contents of the cryovials were then drained on layers of sterile filter paper. The PLBs were placed on a piece of filter paper (Whatman No. 1, 9 cm; Whatman plc, UK) affixed on the recovery medium. The growth recovery was conducted in complete darkness for a week, followed by exposure to dim light using shaded cool white fluorescent lamps (3.4 $\mu molm^{-2}s^{-1}$) for three weeks. The PLBs were then incubated at 25 ± 2ºC under 16 hours photoperiod using cool white fluorescent lamps (Philips TLD, 36 W, 150 $\mu molm^{-2}s^{-1}$) in the subsequent weeks.

Encapsulation-dehydration procedure for PLBs of Dendrobium sonia-28

Three to four mm PLBs were excised and immediately immersed in sodium alginate. Each PLB was aspirated with 50 µL of the alginate medium into a 1 mL pipette, and dropped into culture jars containing 25 mL 0.1 M calcium chloride solution. Beads formed were left to incubate in the solution for 30 minutes with occasional agitation. The beads were then precultured in 0.5 M sucrose for 72 hours on an orbital shaker set at 60 rpm. After the preculture treatment, the beads were dabbed dry on sterile filter papers and dehydrated in hermetically-sealed culture jars containing 50 g heat-sterilised silica gel layered with a piece of sterile filter paper, for four hours. They were then placed in 2 mL cryovials, and immediately immersed in liquid nitrogen for 24 hours. Storage in liquid nitrogen and thawing were omitted for non-cryopreserved beads. Frozen beads were thawed in a 40 ± 2°C water bath for 90 seconds. The thawed cryopreserved and non-cryopreserved beads were placed in liquid recovery medium on an orbital shaker set at 60 rpm. Recovery of both cryopreserved and non-cryopreserved PLBs were conducted over a span of three weeks at 25°C. The recovery procedure was conducted exactly as that conducted in the vitrification treatment.

DNA extraction and amplification from untreated, non-cryopreserved and cryopreserved PLB samples of Dendrobium sonia-28

Five types of samples were selected for genomic DNA amplification and analysis: non-treated PLBs obtained fresh from stock cultures, non-cryopreserved PLBs from both the encapsulation-dehydration and optimised vitrification protocols, and recovering cryopreserved PLBs from both the encapsulation-dehydration and optimised vitrification protocols. Samples were collected from randomly selected plants for each treatment, with the minimal age of each *in vitro* plantlet set at three months. The genomic DNA of the each sample was extracted using the Genomic DNA Mini Kit (Plant; Geneaid Biotech Ltd., Taiwan, Republic of China). The DNA purity was established and calculated using samples diluted 1000 times with 1× Tris-EDTA (TE) buffer through measurements of the samples' absorbance ratios at 260 nm and 280 nm. For the RAPD assays, 15 arbitrary 10-mers (1st Base, Malaysia) were selected from a list of 20 primers that were screened by Khosravi et al. (2009), based on highly scoring polymorphic bands in their molecular analysis of *Dendrobium* Serdang Beauty (Table 3). All chemicals and buffers used in the RAPD and PCR protocols were acquired from Fermentas Life Sciences (Vilnius, Lithuania). Each 20 µL PCR reaction cocktail, in a 200 µL PCR tube (Axygen Inc., California, USA), consisted of: 20 ng of the plant DNA template; one unit of *Taq* DNA polymerase (DreamTaq™ DNA Polymerase, 5 $U\mu L^{-1}$); 0.5 µL of 10 mM dNTP Mix; 2 µL of 10× PCR buffer (10× DreamTaq™ Buffer) containing 100 mM Tris-Hydrochloride (Tris-HCl) at pH 8.8, 500 mM potassium hydrochloride (KCl), 0.8% (v/v) Nonidet P40 and 20 mM magnesium chloride ($MgCl_2$); 1 µL from 10 mM of a single primer; and

nuclease-free distilled deionised water. The DNA amplification was performed using MyCycler™ Thermal Cycler (Bio-Rad Laboratories, Inc., USA), with the following conditions: initial denaturation at 95°C for three minutes; 35 cycles of denaturation at 95°C for 30 seconds, annealing at 5°C below each primer's melting temperature (T_m) for 30 seconds and extension at 72°C for one minute; and a final extension cycle at 72°C for 10 minutes. Genomic DNA was omitted in the negative control reactions to observe for possible sample or reaction contamination. The PCR products were cooled and left at 4°C at the end of the reaction, and finally stored at -41°C prior to electrophoresis.

For electrophoresis, 10 µL of each of the PCR product was mixed with 5 µL of 6× DNA Loading Dye (Fermentas Life Sciences, Vilnius, Lithuania), and loaded into 1.50 × 4.84 mm wells of a 50 mm 1.5% (w/v) agarose gel. The electrophoresis was conducted at 75 V using PowerPac™ Basic Power Supply (Bio-Rad Laboratories, Inc., USA). The electrophoresed agarose gel slab was immersed in 0.5 µgmL^{-1} ethidium bromide (EtBr) for 10 minutes, and rinsed in distilled water for 30 seconds, and then placed on a 302 nm UV transilluminator (Molecular Imager® Gel Doc™ XR+ System with Image Lab™ and Quantity One 1-D Analysis Software, Bio-Rad Laboratories, Inc., USA) for nucleic acid visualisation, photography and analysis. The DNA fragment patterns of each treatment group were evaluated by calculating the Similarity Indices of the groups as compared to the DNA bands of the control plant. The presence or absence of each band was manually scored as '**1**' or '**0**' respectively, and the coefficients of similarity between the treatment and control groups were calculated using the formula below (Nei and Li, 1979; Asnita and Norzulaani, 2006):

$$\textbf{Similarity Index (SI)} = \frac{2N_{xy}}{N_x + N_y}$$

where N_{xy} = number of monomorphic bands between the control and treatment groups

N_x = total number of bands in the control group

N_y = total number of bands in the treatment group

Experimental design

Growth recovery assessments were conducted by observing for visible PLB proliferation or growth after 12 weeks (three months) of post-cryopreservation recovery. Each experiment consisted of nine replicates containing 10 PLBs. Means were analysed through one-way ANOVA and differentiated with Tukey's test, with the probability value set at 0.05.

Conclusion

The RAPD analyses of PLBs of *Dendrobium* sonia-28 obtained from the stock culture, control encapsulation-dehydration and vitrification treatments, and frozen samples from encapsulation-dehydration and vitrification treatments indicated that PLBs that were vitrified and cryopreserved possessed molecular stability when compared to those that were encapsulated, dehydrated and then cryopreserved. However, despite the low regeneration rates, this treatment is considered a success as new PLBs and plantlets of *Dendrobium* sonia-28 were speedily produced from surviving cryopreserved PLBs, displaying the typical totipotency associated with orchid PLBs. The RAPD analyses indicated that the vitrification technique is a safe method of conserving PLBs of *Dendrobium* sonia-28, as the molecular stability of the hybrid was preserved after the cryopreservation treatment. Hence, the vitrification technique can be applied on a large scale for preservation and propagation of the orchid hybrid.

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