TÜBİTAK

Turkish Journal of Botany

http://journals.tubitak.gov.tr/botany/

Research Article

Turk J Bot
(2013) 37: 940-949
© TÜBİTAK
doi:10.3906/bot-1208-35

Refinement of a vitrification protocol for protocorm-like bodies of *Dendrobium* sonia-28

Ranjetta POOBATHY[1], Uma Rani SINNIAH[2], Maziah MAHMOOD[3], Sreeramanan SUBRAMANIAM[1,*]

[1]School of Biological Sciences, Universiti Sains Malaysia, Gelugor, Pulau Pinang, Malaysia
[2]Department of Crop Sciences, Universiti Putra Malaysia, Serdang, Selangor, Malaysia
[3]Department of Biochemistry, Faculty of Biotechnology and Molecular Sciences, Universiti Putra Malaysia, Serdang, Selangor, Malaysia

Received: 22.08.2012 • **Accepted:** 29.04.2013 • **Published Online:** 06.09.2013 • **Printed:** 30.09.2013

Abstract: The hybrid *Dendrobium* sonia-28 is an important ornamental orchid in the Malaysian flower industry. However, it faces the risk of producing heterozygous progenies through conventional seed propagation or somaclonal variation. Cryopreservation is a favoured long-term storage method in the preservation and maintenance of orchids with specific desired traits. This paper presents a successful cryopreservation protocol for protocorm-like bodies (PLBs) of *Dendrobium* sonia-28 through the vitrification technique and the use of ascorbic acid and charcoal. Survival assessments were conducted through growth evaluations and visual and spectrophotometric 2,3,5-triphenyltetrazolium chloride assays. Results of this study, an improvement over the previous protocol applied to this orchid hybrid, showed that the addition of ascorbic acid to the preculture, loading, dehydration, unloading, and regeneration media, coupled with the addition of charcoal to the regeneration medium, helped boost recovery rates of cryopreserved PLBs of *Dendrobium* sonia-28.

Key words: Cryopreservation, *Dendrobium* sonia-28, plant vitrification solution 2, protocorm-like bodies

1. Introduction

Orchidaceae, one of the largest flowering plant families, comprises more than 880 genera and almost 30,000 species, with an estimated 800 species identified and added to the list annually (Nicoletti, 2003; Bektaş et al., 2013). The orchid genus *Dendrobium* is popular in the international floriculture industry due to its floriferous flower sprays; wide spectrum of colours, sizes, and shapes; year-round availability; and long flowering life (Kuehnle, 2007; Khosravi et al., 2009). *Dendrobium* sonia-28, a hybrid resulting from the cross between 2 hybrids, *Dendrobium* Caesar and *Dendrobium* Tomie Drake, is prized for its pink-coloured and good-quality cut flowers. The current orchid clonal propagation scheme is based on the regeneration of newly formed protocorms through sections of protocorms and protocorm-like bodies (PLBs) (Morel, 1974; Saiprasad & Polisetty, 2003). Protocorm cultures can be maintained for an unlimited period of time and at fantastic rates as sectioned and subcultured protocorms regenerate into new protocorm clumps instead of differentiating into buds (Morel, 1974). Hence, protocorms and PLBs are attractive as target tissues for cryopreservation.

Orchid populations are susceptible to extinction due to destructive factors such as climate change, habitat destruction, overharvesting, and reductions in the numbers of natural pollinators (Koopowitz, 2001; Vaasa & Rosenberg, 2004; Machaka-Houri et al., 2012). Hence, there is a need to promote and prioritise orchid conservation, including monitoring and management practices (Işık, 2011; Machaka-Houri et al., 2012). Cryopreservation, a component of ex situ conservation, involves the storage of viable cells at ultralow temperatures (e.g., –196 °C), usually in gas phase or liquid nitrogen (LN) (Benson et al., 2007). The metabolic activities of the cells are assumed to be arrested at such temperatures, hence stabilising the cells for indefinite periods as long as the LN supply is maintained (Panis et al., 1998).

The vitrification process is defined as solidification of a liquid not through crystallisation but rather by an extreme elevation in viscosity during cooling (Fahy et al., 1984; González-Benito et al., 2004). Cellular viscosity is enhanced through the addition of cryoprotective substances at high concentrations, and by removing water from the target explants through evaporative desiccation and/or osmotic dehydration (Benson, 2008). The glycerol-

* Correspondence: sreeramanan@gmail.com

based plant vitrification solution 2 (PVS2) (Sakai et al., 1991) contains 30% glycerol (w/v), 15% ethylene glycol (w/v), and 15% dimethyl sulfoxide (Me_2SO, w/v) in basal culture medium containing 0.4 M sucrose (pH 5.8). PVS2 easily supercools below –100 °C upon rapid cooling and solidifies into a metastable glass at about –115 °C (Sakai et al., 1990). The vitrification technique and its derivatives have been applied in a number of temperate and tropical species and for different explants such as cell suspensions, somatic embryos, and shoot tips (Sakai & Engelmann, 2007).

High recovery rates of cryopreserved explants can be sustained when the recovery media are designed to suppress production of free radicals from photooxidation or provide free radical scavenging elements (Touchell & Walters, 2000; Normah & Makeen, 2008), and this can be achieved through the supplementation of suitable antioxidants and frequent subcultures (Chandel et al., 1996; Normah & Makeen, 2008). Ascorbic acid, also known as vitamin C, is a water soluble antioxidant that regenerates tocopherol or vitamin E from its radical form (Leung et al., 1981; Kamal-Eldin & Appelqvist, 1996; Uchendu et al., 2010). Ascorbic acid is able to directly interact with the superoxide radical ($O_2^{\bullet}$), hydrogen peroxide (H_2O_2), the hydroxyl radical (•OH), and lipid hydroperoxides (Shao et al., 2008; Uchendu et al., 2010). Uchendu et al. (2010) discovered that the application of exogenous tocopherol or ascorbic acid at critical steps of the PVS2 vitrification process significantly increased the regrowth of shoot tips in 2 blackberry cultivars.

The objectives of this study were to refine the vitrification protocol initiated for PLBs of the orchid hybrid *Dendrobium* sonia-28 (Tan et al., 2010) by expanding the preculture, loading, and dehydration parameters, and through the addition of medium additives, i.e. ascorbic acid and charcoal.

2. Materials and methods

2.1. Propagation of plant materials

PLB cultures of *Dendrobium* sonia-28 were initiated by aseptically culturing seeds of the hybrid in semisolid, half-strength Murashige and Skoog (1/2 MS) (Murashige & Skoog, 1962) medium with 2% sucrose, 2.75 g L^{-1} Gelrite™ (DUCHEFA, the Netherlands), and 1 mg mL^{-1} 6-benzylaminopurine (BAP; DUCHEFA). The resulting cultures were chopped into clumps of 2–3 PLBs and subcultured every 4 weeks. The cultures were incubated at 25 ± 2 °C under a 16 h photoperiod using cool white fluorescent lamps (Philips, TLD; 36 W, 150 µmol m^{-2} s^{-1}).

2.2. Preparation of experimental media

All vitrification treatments employed in this study were adapted and modified from the basic vitrification protocol described by Sakai et al. (2008). All media used contained 1/2 MS components. The semisolid preculture medium contained various concentrations of sucrose (0, 0.2, 0.4, 0.6, and 1.0 M; DUCHEFA); it was gelled with 2.75 g L^{-1} Gelrite™, and poured into sterile plastic petri dishes (9 cm; BRANDON). The loading solution contained 0.4 M sucrose and 2.0 M glycerol. The PVS2 consisted of 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) Me_2SO, and 0.4 M sucrose (Sakai et al., 1990), and the unloading solution contained 1.2 M sucrose. The recovery medium consisted of semisolid pour plates (9 cm; BRANDON) of 1/2 MS components with 2% sucrose and 2.75 g L^{-1} Gelrite™. The pH value (CyberScan PC 510 pH/mV/Conductivity/TDS/°C/°F Bench Meter, Eutech Instruments, Singapore) was adjusted to 5.8 prior to autoclaving (STURDY SA-300VFA-F-A505, Sturdy Industrial Co. Ltd., Taiwan).

2.3. Effect of sucrose preculture

To select the best preculture concentration and duration, PLBs of 3–4 mm were precultured in 1/2 MS medium with 0, 0.2, 0.4, 0.6, and 1.0 M sucrose for 24, 48, or 72 h. The precultured PLBs were then placed in 2-mL sterile cryovials (Nalgene Nunc, USA) and immersed in 1.5 mL of loading solution for 20 min at room temperature, with exchange of fresh solution at 10-min intervals. The loading solution was aliquoted out of the cryovials, and the PLBs were then immersed in 1.5 mL of PVS2 at 0 °C for 20 min, with exchange of fresh solution at 10-min intervals. The cryovials were fixed to aluminium cryocanes (5015-0002, Nalgene Nunc). This was followed by direct plunging and storage in LN (–196 °C) for 24 h (MVE Lab 20, MVE Bio-Medical Division, Chart Industries, Inc., USA). Frozen PLBs were thawed in a 40 ± 2 °C water bath for 90 s. After thawing, the PVS2 solution was immediately removed from the cryovials, and PLBs were immersed in 1.5 mL of unloading solution for 20 min at room temperature, with an exchange of fresh solution at 10-min intervals. The PLBs were placed on a piece of filter paper (Whatman No. 1, 5 cm; Whatman, UK) affixed on 1/2 MS semisolid recovery medium and left to incubate in the dark for 3 days, after which the PLBs were transferred onto fresh recovery medium, excluding the filter paper. Storage in LN and thawing were omitted for noncryopreserved PLBs; instead, they were immersed in unloading solution immediately after PVS2 application and were transferred on recovery medium as were the cryopreserved samples. Recovery of both cryopreserved and noncryopreserved PLBs was conducted over a span of 3 weeks at 25 °C. The first week of recovery was conducted in complete darkness, and the second week involved a 1-h light exposure every 24 h under cool white fluorescent lamps (Philips TLD; 36 W, 150 µmol m^{-2} s^{-1}). The PLBs were then incubated at 25 ± 2 °C under a 16-h photoperiod using cool white fluorescent lamps in the third week.

2.4. Effect of dehydration treatment duration

In order to determine the best dehydration period with PVS2 solution, PLBs of 3–4 mm were precultured in semisolid 1/2 MS medium with 0.4 and 1.0 M sucrose for 48 h and 0.6 M sucrose for 72 h (those combinations were selected based on the results of the sucrose preculture trial), followed by immersion in 1.5 mL of loading solution for 20 min. This was followed by dehydration in PVS2 at 0 °C for 0, 10, 20, 30, 40, 50, 60, 80, 100, and 120 min prior to cryostorage. The recovery of both noncryopreserved and cryopreserved PLBs was performed as described above.

2.5. Effect of regeneration conditions and the use of medium additives

All solutions used in all stages of this treatment (preculture, loading, PVS2, unloading, and recovery media) were supplemented with 0.6 mM L-ascorbic acid (R&M Chemicals, UK), the higher end of the effective concentration range reported by Uchendu et al. (2010). Ascorbic acid was filter-sterilised using a 0.45-µm syringe filter (Sartorius Minisart®, NML Syringe Filters) and was added to the autoclave-sterilised media. In this treatment, friable PLBs of *Dendrobium* sonia-28 of 3–4 mm were precultured in semisolid 1/2 MS medium with 0.4 M sucrose for 48 h, followed by immersion in 1.5 mL of a loading solution for 20 min and dehydration in PVS2 at 0 °C for 20, 50, 80, and 100 min. In another test the PLBs were precultured, loaded, and then subjected to dehydration for 50, 80, and 100 min (these combinations were selected based on the results of the ascorbic acid treatment), followed by recovery in medium containing a combination of 0.6 mM ascorbic acid and 2 g L^{-1} charcoal (DUCHEFA). The methodology of the following procedures (loading, dehydration with PVS2, cryopreservation, thawing, unloading, and growth recovery) was conducted as described in the previous section.

2.6. 2,3,5-Triphenyltetrazolium chloride method of assessing survival of cryopreserved and noncryopreserved PLBs of *Dendrobium* sonia-28

All cryopreserved and noncryopreserved PLBs were subjected to a survival assessment through this method (Steponkus & Lanphear, 1967; Verleysen et al., 2004). The PLBs were collected after 3 weeks of recovery and assessed for growth, proliferation, and colour (green) that could be observed with the naked eye. After that, all 10 PLBs in a single replicate were placed in a black-capped culture vial (21 mm × 85 mm), and 2 mL of the TTC buffer was aliquoted into the vial. The solution comprised 0.6% (w/v) 2,3,5-triphenyltetrazolium chloride (TTC; Merck, Germany) and 0.05% (v/v) Tween 20 (R&M Chemicals) in a buffer consisting of 0.05 M of both disodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$) and potassium dihydrogen phosphate (KH_2PO_4) (R&M Chemicals), set at pH 7.4. The culture vials were then incubated in the dark at 27–30 °C for 18–24 h. After the incubation period, the PLBs were visually scanned for surface area stained with formazan using a stereoscopic dissecting microscope (Olympus, Japan), with a spot of redness taken as the minimal indication of PLB survival. The same PLBs were then subjected to the spectrophotometric-TTC assay.

In the spectrophotometric-TTC assay, the PLBs were washed 3 times with 3.5 mL of distilled water. After the final rinse, 7 mL of 95% ethanol was added into each of the culture vials. All the culture vials were then placed in an 85 °C water bath for 1 h to fully extract the formazan. The vials were allowed to cool to room temperature, and the formazan extract in each culture vial was topped up to 7 mL, again using 95% ethanol. The absorbance of the extracts was then read against a blank of 95% ethanol at 490 nm using a spectrophotometer.

2.7. Statistical analyses

The treatments consisted of 9 replicates, each containing 10 PLBs. Means were analysed with 1-way ANOVA and differentiated with Tukey's test. The probability value was set at 0.05.

3. Results

3.1. Effect of sucrose preculture

The control preculture treatment (Table 1; Figure 1) indicated that high sucrose concentrations had a detrimental effect on the PLBs of *Dendrobium* sonia-28, as survival percentages reduced drastically when they were precultured in sucrose concentrations higher than 0.4 M. Cryopreserved PLBs that were precultured for 24 h in sucrose did not survive the effects of cryostorage. However, significant results were obtained from the spectrophotometric-TTC assays when the preculture was conducted for 48 or 72 h (Figure 2). In accordance with these findings, the following sucrose concentrations and preculture durations were selected to proceed with the next stages of the cryopreservation experiment: 0.4 and 1.0 M for 48 h and 0.6 M for 72 h.

Growth in noncryopreserved PLBs occurred in 2 ways: from their previous stage of growth prior to the treatment, or through the proliferation of new PLBs on the mother PLB (Figures 3a and 3b). Most noncryopreserved PLBs bleached or turned white in the first week of recovery but produced new PLB clumps within 3 weeks. Despite initially showing growth and proliferation, cryopreserved PLBs bleached within 3 days of exposure to light in the third week of recovery. No growth was observed for any of the cryopreserved PLBs thereafter.

3.2. Effect of dehydration with PVS2

Observations made in the PVS2 dehydration treatment were similar to those made in the preculture treatment. A 48-h preculture in medium with 1.0 M sucrose resulted in PLBs with a flaccid and soft exterior prior to

Table 1. Effect of various sucrose preculture durations on noncryopreserved PLBs of *Dendrobium* sonia-28. Means with the same letters are not significantly different.

Preculture concentration (M)	Survival percentage (green or proliferating clump)		
	24 h	48 h	72 h
0 (control)	91.1ab	86.7ab	96.7a
0.2	96.3a	88.3ab	98.3a
0.4	62.2c	26.7d	75.0bc
0.6	10.0de	0e	8.3de
1.0	1.1e	0e	0e

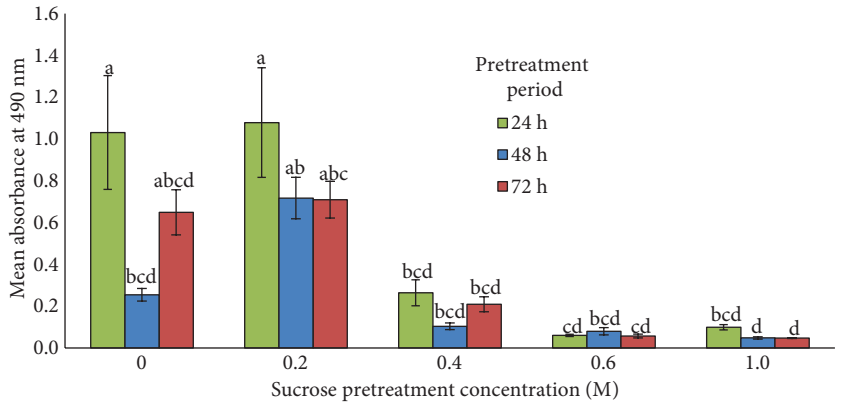

Figure 1. The effect of sucrose preculture concentrations and duration on noncryopreserved PLBs of *Dendrobium* sonia-28, as obtained from spectrophotometric-TTC assay. Error bars represent the standard error of the means. Means with the same letters are not significantly different.

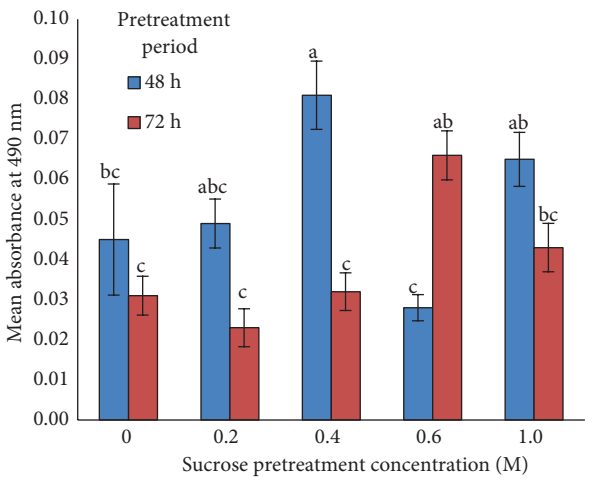

Figure 2. The effect of sucrose preculture concentrations and duration on the cryopreservation of PLBs of *Dendrobium* sonia-28, as obtained from the spectrophotometric-TTC assay after 3 weeks of incubation on the recovery medium. No regeneration was observed for any of the cryopreserved PLBs. Error bars represent the standard error of the means. Means with the same letters are not significantly different.

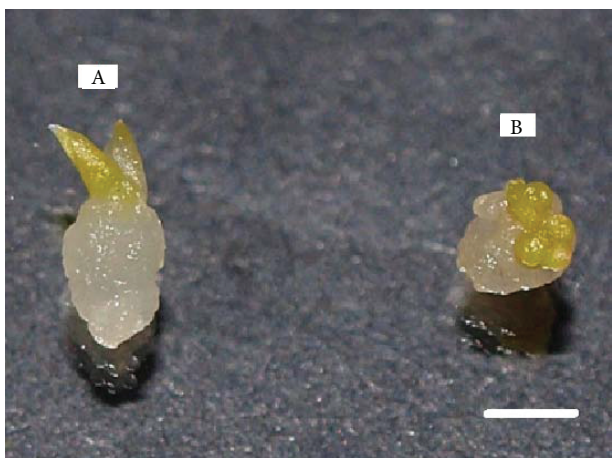

Figure 3. Growth of PLBs that were dehydrated with PVS2 but not stored in LN occurred in 2 ways: a) from their previous stage of growth prior to the treatment or b) through the proliferation of new PLBs on the mother PLB. Scale bar = 2 mm.

cryopreservation. However, it was noted that the PLBs also displayed similar traits when they were dehydrated for more than 60 min in PVS2, irrespective of the sucrose preculture concentration. Furthermore, cryopreserved PLBs that were dehydrated for more than 60 min were the earliest to start dechlorophyllation, with all PLBs turning yellow within 2 days of the recovery treatment.

Data from the various PVS2 dehydration periods tested in the control treatment indicated that PLB survival is more dependent on the preculture concentration used than the dehydration duration itself (Table 2). Visible PLB proliferation was obtained from preculture involving 0.4 M sucrose, but the growth data presented high variability. The highest PLB growth and proliferation rate in the control treatment was obtained when the PLBs were precultured in 0.4 M sucrose followed by 20 min of loading treatment and either 0 min or 40 min of dehydration in PVS2, with both producing 48.0% and 47.0% growth. No significant differences were detected between cryopreserved PLBs that were precultured in 0.4 M sucrose before being subjected to various periods of dehydration (Table 3). However, visible growth of PLBs was obtained, albeit after

Table 2. Effect of various PVS2 dehydration periods on noncryopreserved PLBs of *Dendrobium* sonia-28 after 6 weeks of PLB recovery. Means with the same letters are not significantly different.

PVS2 treatment duration (min)	Survival percentage (green or proliferating clump)		
	Preculture with 0.4 M sucrose (48 h)	Preculture with 0.6 M sucrose (72 h)	Preculture with 1.0 M sucrose (48 h)
0	48.0a	0.0d	3.0d
10	19.0bcd	28.0abc	0d
20	41.0ab	13.0cd	0d
30	2.0d	2.0d	0d
40	47.0a	6.0cd	0d
50	18.0cd	8.0cd	0d
60	14.0cd	2.0d	2.0d
80	7.0cd	1.0d	0d
100	20.0bcd	4.0d	0d
120	0d	12.0cd	0d

Table 3. Effect of various PVS2 dehydration periods on the survival of cryopreserved PLBs of *Dendrobium* sonia-28 precultured in 0.4 M sucrose for 48 h, as determined by visual-TTC and growth evaluations after 6 weeks of PLB recovery.

PVS2 treatment duration (min)	Survival percentage (visual-TTC)	Survival percentage (green or proliferating clumps of PLBs)
0	2.2	0
10	0	0
20	1.1	4.4
30	1.1	0
40	0	1.1
50	8.9	0
60	0.0	1.1
80	5.6	0
100	1.1	2.2
120	0	2.2

5 weeks of recovery, with 50 and 80 min of dehydration; this produced 2 of the highest growth rates (8.9% and 5.6%, respectively), as assessed through visual-TTC analysis.

All cryopreserved PLBs bleached within 1 day of being exposed to light at the end of 1 week of incubation in the dark. No visible growth was observed for up to 3 weeks of the recovery period, despite incubation under dim light conditions. However, new PLB growth was observed on some cryopreserved PLBs beyond the fifth week of recovery. The growth of the cryopreserved PLBs then resumed, although slowly, like that observed in untreated PLBs, beginning from the sixth week of recovery. The growth could not be sustained, and almost all PLBs that displayed growth earlier bleached or turned brown with a single transfer from old medium to fresh medium.

The final growth evaluation data, obtained after 6 weeks of growth recovery treatment, showed that 20 min of dehydration following preculture in 0.4 M sucrose and 20 min of loading treatment produced the highest recovery (4.4%) (Table 3). The following dehydration periods were selected from the visual-TTC and growth evaluation data to proceed with in the next study: 20, 50, 80, and 100 min.

3.3. Effect of regeneration conditions and the use of medium additives

Observations made in this treatment for both cryopreserved and noncryopreserved PLBs were similar to those made in the dehydration treatments. Survival percentages obtained from the control treatment involving the use of 0.6 mM ascorbic acid in all media did not differ significantly from each other, except when 20 min of dehydration was applied to the PLBs (Table 4). No growth was observed in cryopreserved PLBs that were dehydrated for 20 min in PVS2 after 12 weeks of recovery; hence, the treatment was eliminated from the experiment. The combination of ascorbic acid and charcoal boosted callus proliferation in all PLBs treated in the treatment, regardless of their cryopreservation status (Table 5). This observation was unique as no callus proliferation was observed in the earlier cryopreservation and control treatments. Calli produced were either white or light yellow in colour (Figures 4a and 4b) and formed globular clumps on the treated PLBs. The addition of ascorbic acid and charcoal boosted recovery percentages in the control group when dehydration was conducted for either 50 min or 80 min.

Table 4. Effect of 0.6 mM ascorbic acid and various PVS2 dehydration periods on noncryopreserved and cryopreserved PLBs of *Dendrobium* sonia-28, as determined by growth evaluations after 6 and 12 weeks of PLB recovery. Means with the same letters are not significantly different.

PVS2 treatment duration (min)	Survival percentage (green or proliferating clump)		
	–LN (12 weeks recovery)	+LN (6 weeks recovery)	+LN (12 weeks recovery)
20	60.0a	12.0b	0
50	25.6b	14.4b	2.2
80	16.7b	8.9b	2.2
100	12.2b	12.2b	3.3

Table 5. Effect of 0.6 mM ascorbic acid and charcoal and various PVS2 dehydration periods on noncryopreserved and cryopreserved PLBs of *Dendrobium* sonia-28, as determined by growth evaluations after 12 weeks of PLB recovery. Means with the same letters are not significantly different.

LN	Dehydration period (min)	Percentage observation	
		PLB growth	Callus formation
–LN	50	30.0a	88.9pq
	80	17.0ab	85.0q
	100	2.0c	89.0pq
+LN	50	16.0abc	100.0p
	80	2.0c	96.0pq
	100	8.0bc	100.0p

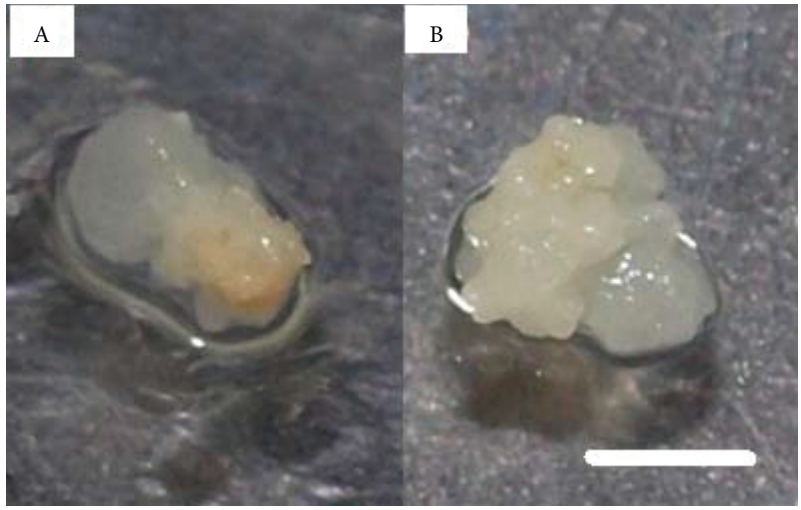

Figure 4. Globular callus formation on cryopreserved and noncryopreserved PLBs subjected to treatments involving the use of media with ascorbic acid and charcoal. Calli formed were either a) light yellow or b) white in colour. Scale bar = 2 mm.

The data obtained in the cryopreservation treatments were the first signs of sustainable growth obtained from any cryopreservation exercise in this research. When ascorbic acid was added in all media used, treatments involving 50, 80, or 100 min of dehydration in PVS2 followed by cryostorage produced 8.9%–14.4% survival 6 weeks into the recovery treatment, but the growth was not sustainable. The use of charcoal, however, promoted sustainable growth of PLBs, as recovery percentages obtained from 50, 80, and 100 min of dehydration at the sixth week were maintained right up to the 12-week mark. The cryopreserved PLBs were able to produce new PLBs that proliferated into plantlets within 6 months of the recovery period (Figure 5). No morphological differences were detected between the recovered cryopreserved explants and plantlets from the stock culture.

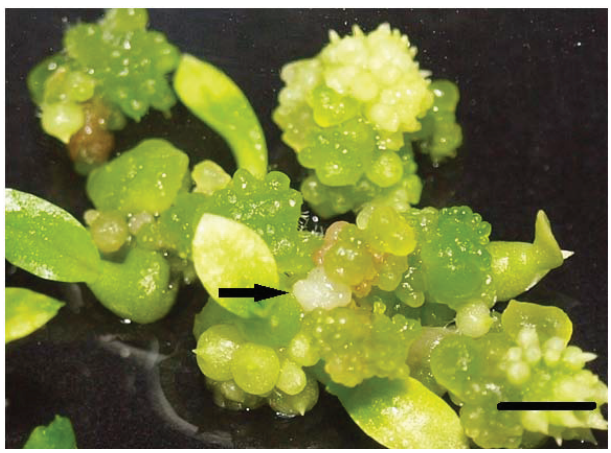

Figure 5. The in vitro proliferation of PLBs and plantlets from a single cryopreserved PLB (arrow) of *Dendrobium* sonia-28 after 6 months of culture on semisolid 1/2 MS medium with 2% (w/v) sucrose and 0.2% (w/v) charcoal. Scale bar = 1 cm.

4. Discussion

4.1. Effect of preculture

Preculture involves the culture of the explants for a short period of time ranging from several hours to days after excision. The cells undergo mild osmotic stress during the preculture, causing metabolic changes in the cells while enhancing chilling and desiccation tolerance (Engelmann et al., 2008). When sugar preconditioning is conducted using high sugar concentrations, for example over 1.0 M, sugar itself may become toxic to the mass of cells, especially with prolonged preculture periods (Wu et al., 2003; Lambardi et al., 2008). This was observed when PLBs of *Dendrobium* sonia-28 were precultured for various durations in sucrose at concentrations above 0.6 M and 0.8 M in the control and freezing treatments. However, results obtained from both treatments suggest that the low survival percentages in the control treatments originated from the sugar concentrations used in the preculture rather than the duration of the preculture.

The best results in the encapsulation-vitrification of PLBs of *Dendrobium candidum* Wall. ex Lindl. were obtained when the PLBs were subjected to a 5-day preculture in 0.75 M sucrose medium with BAP and 1-naphthalene acetic acid (Yin & Hong, 2009). In a preliminary study by Tan et al. (2010), the best viability rate in the cryopreservation of *Dendrobium* sonia-28 was achieved when PLBs of 3–4 mm were precultured in semisolid 1/2 MS media with 0.6 M sucrose and dehydrated in PVS2 at 0 °C for 20 min with no regeneration reported

by the team. In the cryopreservation of *Dendrobium* Bobby Messina by vitrification, the best viability rate was obtained when PLBs of 3–4 mm were precultured in either 0.6 M sucrose or 1.2 M sorbitol (Antony et al., 2010).

Preculture was necessary in the cryopreservation of *Doritaenopsis* suspension culture, as cells that were not precultured showed very low TTC stainability (6.1%). Cells that were precultured for a week in 0.3 or 0.4 M sucrose appeared bleached and damaged and displayed low TTC stainability, indicating that the suspended cells were not tolerant to high sucrose concentrations (Tsukazaki et al., 2000). This was also observed in this research, as PLBs that were precultured in sucrose at concentrations above 0.6 M in the control treatment bleached within a few days of light exposure.

4.2. Effect of dehydration with PVS2

The most common approach in plant cryopreservation involves the treatment of samples with PVS2 for 30 to 90 min. The most important parameter to optimise at this stage is the PVS2 incubation period, as toxicity could occur in the samples due to extended incubations in the cryoprotectant (Lambardi et al., 2008). Tiau et al. (2009) discovered that PLBs of *Dendrobium* sonia-28 of 2–4 mm gave the highest viability when PLBs were precultured in 0.25 M sucrose, followed by dehydration in PVS2 at 0 °C for 20 min, with no regeneration reported.

Cryopreserved PLBs of *Bratonia* had to be dehydrated for 1 h in modified PVS2 with PEG instead of ethylene glycol to boost recovery percentages after thawing up to 20.4% (Popova et al., 2010). It was also observed in the cryopreservation of *Phaius tankervilleae* that the highest cryopreserved seed survival rate (79%) was obtained when the seeds were exposed to PVS2 for 60 min, while longer exposure times of up to 120 min caused a slight decrease in survival rates (Hirano et al., 2009). This was observed in this study, as well, as cryopreserved PLBs of *Dendrobium* sonia-28 that were dehydrated for more than 60 min were the earliest to start dechlorophyllation, with all PLBs yellowing within 2 days of the growth recovery treatment.

4.3. Effect of regeneration conditions and the use of medium additives

Uchendu et al. (2010) discovered that exogenous ascorbic acid was readily taken up by blackberry shoot tips and that cryopreserved shoot tips treated with ascorbic acid at the preculture, loading, rinsing, or regrowth stages had significantly higher regrowth rates. In this study, the success in regenerating cryopreserved PLBs of *Dendrobium* sonia-28 can be attributed to the addition of ascorbic acid in all media used in the procedure and the addition of charcoal in the recovery stage of the protocol. Activated charcoal was reported to assist in recovery after cryopreservation by reducing necrogenesis (Dussert et al., 1992) and the adsorption of toxic substances produced by freeze-injured cells (Kuriyama et al., 1990).

Cryopreserved PLBs of *Dendrobium* sonia-28 displayed high sensitivity to light exposure, as they bleached within 2–3 days of light exposure. The PLBs had to be gradually exposed to plant tissue culture light conditions in stages of darkness, dim lighting, and normal lighting conditions. *Bratonia* protocorms that were cultured in the dark for 1 month after thawing remained green but underwent bleaching within 3–5 days of light exposure (Bukhov et al., 2006). Bukhov et al. (2006) observed that the noncyclic electron transport was strongly inhibited in protocorms after the freeze-thaw cycle in the vitrification of *Bratonia* protocorms. The team discovered that cryopreserved *Bratonia* protocorms lost their ability to photo-oxidise the primary donor of PSI, pigment 700, within 24 h of recovery culture. Hence, results obtained in the cryopreservation of PLBs of *Dendrobium* sonia-28 indicated that the freezing and thawing cycles could have damaged some components of the photosynthetic system of the PLBs.

Growth of cryopreserved PLBs was always observed as new PLB growth on the treated PLB (Figure 3b) rather than the continuation of growth of the treated PLB. This could be attributed to the preservation of single cells or clumps of cells within the PLB, rather than the entire organ. Hence, successfully cryopreserved cells could have taken time to grow and expand, with growth only visible to the naked eye from the fifth week onwards. Cryopreservation treatments involving the use of ascorbic acid and charcoal indicated that surviving cryopreserved PLBs of *Dendrobium* sonia-28 underwent a lag phase of about 5–6 weeks before new PLB growth was visible. This was not observed in noncryopreserved PLBs, which resumed growth almost immediately after the treatment. Previous studies have indicated that cryopreserved cells were slower in their regrowth, generally having a lag period of about 3–5 days (Nishizawa et al., 1993; Shibli et al., 2001; Wang et al., 2002).

It was observed in the cryopreservation treatment involving the use of ascorbic acid that surviving cryopreserved PLBs of *Dendrobium* sonia-28 generally produced heavy callus proliferation that differentiated into new PLBs. The calli were mostly globular in shape and nonfriable, and were light yellow, white, or light brown in colour (Figure 3). Noncryopreserved PLBs in the control treatment produced calli that were able to differentiate into new PLBs within 3 weeks of recovery culture. Callus proliferation was rarely observed in earlier cryopreservation treatments that did not involve the use of ascorbic acid. Zhao et al. (2008) observed that PLB and plant regeneration of *Dendrobium candidum* Wall ex Lindl. initiated from 3 types of callus that formed on explants that were subcultured on optimised medium for 20 days.

Some conservation centres consider 20% recovery as sufficient for long-term preservation (Golmirzaie & Panta, 2000; González-Benito et al., 2004), while others advocate

a survival rate of above 40% (Reed et al., 2000; Reed, 2001; González-Benito et al., 2004). Germplasm conservation should involve either modification to the protocol for each genotype or group of genotypes to maximise recovery percentages, or storage of more explants using a nonoptimal protocol in order to compensate for low recovery (González-Benito et al., 2004). 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.

5. Conclusions

The best survival rate in the cryopreservation treatment (16.0% regeneration) was obtained when PLBs of *Dendrobium* sonia-28 of 3–4 mm were precultured in semisolid 1/2 MS medium with 0.4 M sucrose for 48 h, placed in a loading solution for 20 min, dehydrated for 50 min at 0 °C in PVS2, stored in LN for 24 h, thawed in a 40 ± 2 °C water bath for 90 s, placed in an unloading solution for 20 min, and regenerated in semisolid 1/2 MS medium containing 0.2% (w/v) charcoal, with all media supplemented with 0.6 mM ascorbic acid. The PLBs were gradually exposed to light (darkness in the first week of recovery, and exposure to 3.4 μmol m^{-2} s^{-1} for the subsequent 3 weeks and 150 μmol m^{-2} s^{-1} thereafter).

Acknowledgements

The authors wish to thank the Universiti Sains Malaysia Research University Grant Scheme (USM-RU) and the National Science Fellowship (NSF) for supporting this study.

References

Antony JJJ, Chan LK, Xavier R, Uma Rani S & Sreeramanan S (2010). Preliminary study on cryopreservation of *Dendrobium* Bobby Messina protocorm-like bodies by vitrification. *African Journal of Biotechnology* 9: 7063–7070.

Bektaş E, Cüce M & Sökmen A (2013). In vitro germination, protocorm formation, and plantlet development of *Orchis coriophora* (Orchidaceae), a naturally growing orchid species in Turkey. *Turkish Journal of Botany* 37: 336–342.

Benson EE (2008). Cryopreservation theory. In: Reed BM (ed.) *Plant Cryopreservation: A Practical Guide*, pp. 15–32. Heidelberg: Springer.

Benson EE, Harding K & Day JG (2007). Algae at extreme low temperatures: the cryobank. In: Seckbach J (ed.) *Algae and Cyanobacteria in Extreme Environments*, pp. 365–383. Berlin: Springer.

Bukhov NG, Popova EV & Popov AS (2006). Photochemical activities of two photosystems in *Bratonia* orchid protocorms cryopreserved by vitrification method. *Russian Journal of Plant Physiology* 53: 793–799.

Chandel KPS, Chaudhury R & Radhamani J (1996). Cryopreservation of embryos/embryonic axes—a novel method for the long-term conservation of recalcitrant seed species. In: Normah MN, Narimah MK & Clyde MM (eds.) *In Vitro Conservation of Plant Genetic Resources*, pp. 53–71. Kuala Lumpur: Percetakan Watan Sdn. Bhd.

Dussert S, Mauro MC & Engelmann F (1992). Cryopreservation of grape embryogenic cell suspensions 2: influence of post-culture conditions and application to different strains. *Cryoletters* 13: 15–22.

Engelmann F, Gonzalez-Arnao MT, Wu Y & Escobar R (2008). The development of encapsulation dehydration. In: Reed BM (ed.) *Plant Cryopreservation: A Practical Guide*, pp. 59–75. Berlin: Springer.

Fahy GM, MacFarlane DR, Angell CA & Meryman HT (1984). Vitrification as an approach to cryopreservation. *Cryobiology* 21: 407–426.

Golmirzaie AM & Panta A (2000). Advances in potato cryopreservation at CIP. In: Engelmann F & Takagi H (eds.) *Cryopreservation of Tropical Plant Germplasm: Current Research Progress and Application*, pp. 250–254. Tsukuba, Japan: International Research Centre for Agricultural Sciences; Rome: IPGRI.

González-Benito ME, Clavero-Ramírez I & López-Aranda JM (2004). The use of cryopreservation for germplasm conservation of vegetatively propagated crops. *Spanish Journal of Agricultural Research* 2: 341–351.

Hirano T, Godo T, Miyoshi K, Ishikawa K, Ishikawa M & Mii M (2009). Cryopreservation and low-temperature storage of seeds of *Phaius tankervilleae*. *Plant Biotechnology Reports* 3: 103–109.

Işık K (2011). Rare and endemic species: why are they prone to extinction? *Turkish Journal of Botany* 35: 411–417.

Kamal-Eldin A & Appelqvist LA (1996). The chemistry and antioxidant properties of tocopherols and tocotrienols. *Lipids* 31: 671–701.

Khosravi AR, Kadir MA, Kadzemin SB, Zaman FQ & De Silva AE (2009). RAPD analysis of colchicine induced variation of the *Dendrobium* Serdang beauty. *African Journal of Biotechnology* 8: 1455–1465.

Koopowitz H (2001). *Orchids and Their Conservation*. London: Batsford.

Kuehnle AR (2007). Orchids, *Dendrobium*. In: Anderson NO (ed.) *Flower Breeding and Genetics*, pp. 539–560. Berlin: Springer.

Kuriyama A, Watanabe K, Ueno S & Mitsuda H (1990). Effect of post-thaw treatment on the viability of cryopreserved *Lavandula vera* cells. *Cryoletters* 11: 171–178.

Lambardi M, Ozudogru EA & Benelli C (2008). Cryopreservation of embryogenic cultures. In: Reed BM (ed.) *Plant Cryopreservation: A Practical Guide*, pp. 177–210. Berlin: Springer.

Leung H, Vang MJ & Mavis RD (1981). The co-operative interaction between vitamin E and vitamin C in suppression of peroxidation of membrane phospholipids. *Biochimica et Biophysica Acta* 664: 266–272.

Machaka-Houri N, Al-Zein MS, Westbury DB & Talhouk SN (2012). Reproductive success of the rare endemic *Orchis galilaea* (Orchidaceae) in Lebanon. *Turkish Journal of Botany* 36: 677–682.

Morel GM (1974). Clonal multiplication of orchids. In: Withner CL (ed.) *The Orchids: Scientific Studies*, pp. 169–222. Toronto: John Wiley and Sons.

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

Nicoletti B (2003). Number of orchids. In: Elert G (ed.) *The Physics Factbook. An Encyclopedia of Scientific Essays*. Available at http://hypertextbook.com/facts/2003/BiancaNicoletti.shtml.

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

Normah MN & Makeen AM (2008). Cryopreservation of excised embryos and embryonic axes. In: Reed BM (ed.) *Plant Cryopreservation: A Practical Guide*, pp. 211–240. Berlin: Springer.

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

Popova E, Bukhov N, Popov A & Kim HH (2010). Cryopreservation of protocorm-like bodies of the hybrid orchid *Bratonia* (*Miltonia flavescens* × *Brassia longissima*). *Cryoletters* 31: 426–437.

Reed BM (2001). Implementing cryogenic storage of clonally propagated plants. *Cryoletters* 22: 97–104.

Reed BM, Denoma J & Chang Y (2000). Application of cryopreservation protocols at a clonal genebank. In: Engelmann F & Takagi H (eds.) *Cryopreservation of Tropical Plant Germplasm: Current Research Progress and Application*, pp. 246–249. Tsukuba, Japan: International Research Centre for Agricultural Sciences; Rome: IPGRI.

Saiprasad GVS & Polisetty R (2003). Propagation of three orchid genera using encapsulated protocorm-like bodies. *In Vitro Cellular & Developmental Biology - Plant* 39: 42–48.

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

Sakai A, Hirai D & Niino T (2008). Development of PVS-based vitrification and encapsulation–vitrification protocols. In: Reed BM (ed.) *Plant Cryopreservation: A Practical Guide*, pp. 33–57. New York: Springer.

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

Sakai A, Kobayashi S & Oiyama I (1991). Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb.) by a simple freezing method. *Plant Science* 74: 243–248.

Shao HB, Chu LY, Lu ZH & Kang CM (2008). Primary antioxidant free radical scavenging and redox signaling pathways in higher plant cells. *International Journal of Biological Sciences* 4: 8–14.

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

Steponkus PL & Lanphear FO (1967). Refinement of the triphenyl tetrazolium chloride method of determining cold injury. *Plant Physiology* 42: 1423–1426.

Tan HH, James J, Advina J, Ranjeeta P, Pavallekoodi G & Sreeramanan S (2010). A novel approach for preliminary PVS2 vitrification optimization parameters of *Dendrobium* sonia-28 orchid with Evan Blue staining. *Advances in Environmental Biology* 4: 284–290.

Tiau KH, Xavier R, Chan LK & Sreeramanan S (2009). An assessment of early factors influencing the PVS2 vitrification method using protocorm-like bodies of *Dendrobium* sonia 28. *American-Eurasian Journal of Sustainable Agriculture* 3: 280–289.

Touchell D & Walters C (2000). Recovery of embryos of *Zizania palustris* following exposure to liquid nitrogen. *Cryoletters* 21: 261–270.

Tsukazaki H, Mii M, Tokuhara K & Ishikawa K (2000). Cryopreservation of *Doritaenopsis* suspension culture by vitrification. *Plant Cell Reports* 19: 1160–1164.

Uchendu EE, Leonard SW, Traber MG & Reed BM (2010). Vitamins C and E improve regrowth and reduce lipid peroxidation of blackberry shoot tips following cryopreservation. *Plant Cell Reports* 29: 25–35.

Vaasa A & Rosenberg V (2004). Preservation of the rare terrestrial orchids *in vitro*. *Acta Universitatis Latviensis* 676: 243–676.

Verleysen H, Samyn G, Van Bockstaele E & Debergh P (2004). Evaluation of analytical techniques to predict viability after cryopreservation. *Plant Cell, Tissue and Organ Culture* 77: 11–21.

Wang Q, Gafny R, Sahar N, Sela I, Mawassi M, Tanne E & Perl A (2002). Cryopreservation of grapevine (*Vitis vinifera* L.) embryogenic cell suspensions by encapsulation-dehydration and subsequent plant regeneration. *Plant Science* 162: 551–558.

Wu YJ, Huang XL, Xiao JN, Li J, Zhou MD & Engelmann F (2003). Cryopreservation of mango (*Mangifera indica* L.) embryogenic cultures. *Cryoletters* 24: 303–314.

Yin M & Hong S (2009). Cryopreservation of *Dendrobium candidum* Wall. ex Lindl. protocorm-like bodies by encapsulation-vitrification. *Plant Cell, Tissue and Organ Culture* 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 Cellular & Developmental Biology - Plant* 44: 178–185.