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Effect of PVS2 vitrification on *Brassidium s*hooting star orchid using protocorm-like bodies (PLBs)

Jessica Jeyanthi James Antony[1], Hazirah Burkhan[1], Uma Rani Sinniah[2], Ranjetta Poobathy[1], Sreeramanan Subramaniam[1*]

[1]School of Biological Sciences, Universiti Sains Malaysia, 11800 Gelugor, Pulau Pinang, Malaysia
[2]Department of Crop Science, Faculty of Agriculture, Universiti Putra Malaysia, 43400, Serdang, Selangor Darul Ehsan, Malaysia

[*]Corresponding author: sreeramanan@usm.my; sreeramanan@gmail.com

Abstract

A cryopreservation procedure was developed to preserve protocorm-like bodies (PLBs) of *Brassidium* Shooting Star using the PVS2 vitrification technique. The optimised protocol involved the preculture of 3-4mm PLBs in half-strength Murashige and Skoog (MS) semi-solid medium supplemented with 0.8M sucrose, followed by dehydration in PVS2 solution for 20 minutes at 0^{o}C, prior to storage in liquid nitrogen. The viability of non-cryopreserved and cryopreserved PLBs was determined by the 2,3,5-triphenyltetrazolium chloride (TTC) assay, after two weeks of recovery. The chlorophyll contents, total soluble protein and peroxidase activities of both non-cryopreserved and cryopreserved PLBs were assayed after three weeks of recovery. The results from the biochemical analyses indicated that control PLBs produced the highest viability, followed by treatment on non-cryopreserved PLBs (-LN) and cryostored PLBs (+LN), except in the peroxidase activity assay. The peroxidase activity was detected as the highest in cryostored PLBs followed by treated but non-cryopreserved PLBs, and control PLBs.

Keywords: Cryopreservation; PVS2; orchid; Protocorm-like bodies; vitrification.
Abbreviations: ABA_Absisic-Acid, BAP_Benzylaminopurine, DMSO_Dimethyl sulfoxide, MS_Murashige and Skoog (1962) medium, PLBs_Protocorm-like bodies, PVS2_Plant Vitrification Solution 2.

Introduction

Orchidaceae, the largest family of flowering plants, represents approximately 25,000 species (Yu and Xu, 2007). A total of 6,800 orchid species were found in Tropical Asia, with over 1,000 wild species located in Malaysia alone (Yong and Chua, 1990; Cribb et al., 2003). Problems associated with the maintenance of large *in vitro* collections include time consumption and risks of both contamination and somaclonal variation (Withers, 1987; Cribb et al., 2003; Thammasiri and Soamkul, 2006). Currently, only cryopreservation offers a long-term conservation option, requiring minimum space and maintenance (Sakai et al., 1990; Thammasiri and Soamkul, 2006; Sakai et al., 2008). Two strategies are presently applied in plant cryopreservation experiments: the traditional controlled-rate cooling method (cryopreservation in the presence of ice) and the vitrification technique (cryopreservation in the absence of ice). The controlled-rate freezing method involves the use of penetrating colligative cryoprotectants, precise cooling rate control, ice nucleation and freeze-induced cellular dehydration. The vitrification technique increases cellular viscosity and induces the formation of a thermodynamically metastable glass in cells during cryostorage through the use of highly concentrated penetrating cryoprotectants (dimethyl sulfoxide or DMSO), or by concentrating cell solutes through evaporative desiccation and osmotic dehydration (Benson and Bremner, 2004). Plant cryopreservation experiments require optimal tissue and treatment conditions in order to achieve high post-cryopreservation survival rates. Protocorm-like bodies (PLBs) are good targets for cryopreservation as they are a reliable source of potentially regenerable tissues (Ishikawa et al., 1997; Saiprasad and Polisetty, 2003; Yin et al., 2011). More than one billion plants can be obtained from a single protocorm bud in only nine months, indicating a high regeneration potential of a single explant. The propagation of orchids *in vitro* still relies on the culture of explants to produce protocorm-like bodies (PLBs), which are structurally and functionally similar to protocorms (Cherevchenko and Kushnir, 1986; Bukhov et al., 2006). The 2,3,5-triphenyltetrazolium chloride assay is qualitative for large tissues and organs when visualised under a microscope (Pellett and Heleba, 1998) or analysed with a spectrophotometer (Harding and Benson, 1995). Although destructive to the target tissues (Pellett and Heleba, 1998), the assay is often used for embryos and embryonic axes (Normah and Makeen, 2008) for a speedy assessment of the propagule's viability following cryopreservation. *Brassidium* Shooting Star, placed in the *Oncidium* genus, is a hybrid resulting from a cross between *Oncidium* and *Brassia* (*Oncidium wentworthianum* x *Brassia arcuigera* x *Brassia gireoudiana*). This hybrid has high potential in terms of marketability. This study was conducted to establish a method for preserving PLBs of *Brassidium* Shooting Star orchid through the optimisation of various parameters involved in using the PVS2 method. Many methods are tested in the cryopreservation of various plant genotypes due to the genotypic variations displayed by the different plant species as well as hybrids, resulting in different survival levels.

Hence, the objectives of this study were to establish a cryopreservation protocol using the PVS2 method for this orchid hybrid, followed by survival assessments using biochemical and physiological assays.

Results

Effect of PLB size and preculture with sucrose

One to two mm PLBs produced low TTC absorbance values for both cryopreserved and control samples when compared to 3-4mm PLBs (Figs. 1, 2), following preculture in 0.5M sucrose. Hence, 3-4mm PLBs were selected for further treatment. The TTC absorbance values obtained from extracts of both cryopreserved and non-cryopreserved PLBs increased when the sucrose concentration in the preculture medium increased from 0 to 0.4M (Figs. 3, 4), indicating higher PLB survival, especially in PLBs that were not cryopreserved (Fig. 4). The values then decreased when the PLBs were precultured in 0.6M sucrose. However, an absorbance value of 0.295 was recorded when the PLBs were precultured in 0.8M sucrose prior to cryopreservation (Fig. 3), the highest value observed for the cryopreserved PLBs.

Effect of Treatment with PVS2

In the dehydration treatment, the viability of cryopreserved PLBs was lowest when the PVS2 was applied for 5 minutes at 0°C (0.236). The highest viability (0.269) was obtained when the cryopreserved PLBs were dehydrated in PVS2 for 20 minutes (Fig. 5). Although no significant differences were detected between the different dehydration periods in the treatment, a 20-minute immersion in PVS2 was selected as it produced the highest PLB survival value.

Chlorophyll content

The chlorophyll contents of non-cryopreserved and cryopreserved PLBs were determined by spectrophotometry (Harborne, 1973). It was observed that the control PLBs (stock culture) contained the highest total chlorophyll content (Fig. 6). It was also observed that the total chlorophyll *b* content of PLBs in all treatments was higher than the chlorophyll *a* content. The total chlorophyll *a* and chlorophyll *b* contents in cryopreserved PLBs were recorded at 0.3619 µg/ml and 0.4304 µg/ml respectively (Fig. 6), and at 0.3878 µg/ml and 0.4930 µg/ml respectively (Fig. 6) for non-cryopreserved PLBs. The total chlorophyll *a* and *b* contents in control PLBs were recorded at 0.6533 µg/ml and 1.0627 µg/ml respectively (Fig. 6), the highest values in the treatment. All PLBs subjected to cryopreservation bleached and displayed symptoms of hyperhydricity.

Total Soluble Protein

Cryopreserved PLBs produced the lowest total soluble protein content, while the control PLBs produced the highest total soluble protein content (Fig. 7). The total soluble protein contents in cryopreserved PLBs, non-cryopreserved PLBs and control PLBs were 11 µg/ml, 37.667 µg/ml and 61µg/ml respectively (Fig. 7).

Peroxidase Activity

The peroxidase activities of cryopreserved PLBs, non-cryopreserved PLBs, and control PLBs (Fig. 8) were recorded at 855.727 U/mg, 261.029 U/mg and 126.571 U/mg respectively (Fig. 8).

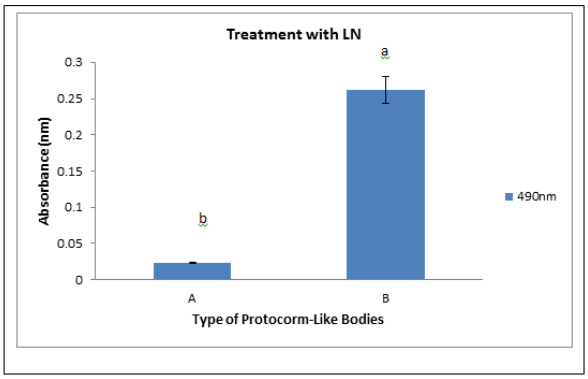

Fig 1. Effect of 0.5M sucrose preculture on cryopreserved PLBs (A: 1-2mm; B: 3-4mm). Error bars represent the standard deviation of means.

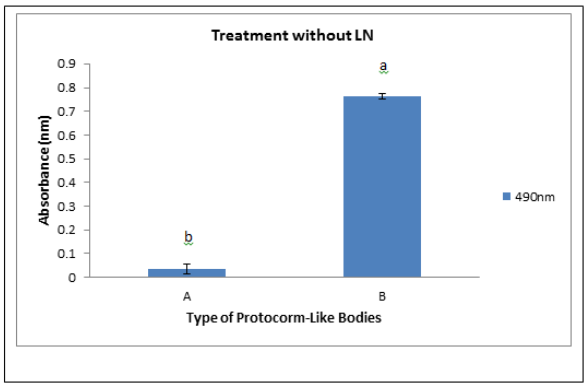

Fig 2. Effect of 0.5M sucrose preculture on non-cryopreserved PLBs (A: 1-2mm; B: 3-4mm). Error bars represent the standard deviation of means.

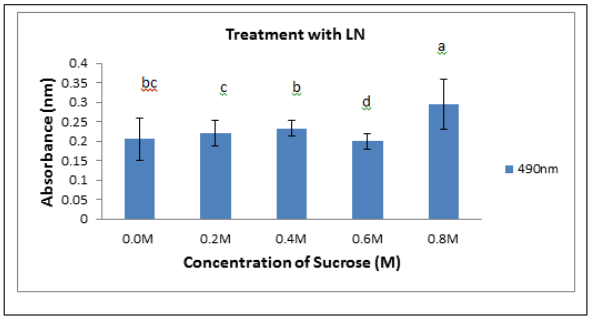

Fig 3. Effect of various concentrations of sucrose preculture on 3-4mm cryopreserved PLBs. Error bars represent the standard deviation of means.

Discussion

It was observed that the preculture of plant materials in medium containing sucrose, followed by the use of cryoprotectants, is integral in improving post-thaw survival rates (Yamada et al., 1991; Niino et al., 1992). The beneficial effect of sucrose in cryopreservation could be attributed to two effects (Steponkus et al., 1992). Firstly, sucrose, similar to other osmotically active substances (e.g. mannitol,

sorbitol), has an osmotic dehydration effect during treatment, leading to reduced water content in the tissue (Tanaka et al., 2004). Sucrose is also able to penetrate the cells (Dumet et al., 1993), proven through histological observations of intracellular accumulation of starch during preculture (Gonzalez-Arnao et al., 1993). Furthermore, sucrose is a natural component of cells, hence the recommendation as a cryoprotectant as it possesses no toxicity even at relatively high concentrations (Dumet et al., 1993). Sucrose is also used as carbon source in media. Sucrose pretreatments conducted for a period of time prior to cryopreservation induces physiological changes in the specimen that favours freeze-tolerance and post-freezing survival (Withers, 1991). Increased sucrose concentrations in pretreatment media could boost the survival of cryopreserved cells and meristems (Uragami et al., 1990; Niino et al., 1992; Niino and Sakai, 1992; Matsumoto et al., 1994; Lurswijidjarus and Thammasiri, 2004). Sucrose preculture induces sugar accumulation, reduces water content and prevents ice crystallization in cells. Steponkus et al. (1992) reported that the accumulation of sucrose within plant tissues contributes to their viability when freezable water is removed to the point of reaching a glassy state during vitrification in the presence of liquid nitrogen. Thus, sucrose preculture assisted in enhancing the tolerance of PLBs of *Brassidium* Shooting Star against the PVS2 treatment. Successful cryopreservation relies on avoidance of lethal intracellular freezing which occurs during rapid cooling in liquid nitrogen or excess osmotic stresses during treatment with PVS2 (Pandey et al., 2008; Sakai et al., 2008). Plant tissues targeted for preservation have to be sufficiently dehydrated to avoid intracellular ice formation and thus vitrify upon rapid cooling in liquid nitrogen (Pandey et al., 2008). Water removal plays a central role in preventing freezing injury and in maintaining post-thaw viability. It was observed that non-cryopreserved PLBs showed higher absorbance readings and cellular viability than those which had been cryopreserved, regardless of the different dehydration periods using PVS2. This occurrence can be attributed to the fact that non-cryopreserved PLBs were not subjected to any freezing damage. Thus, more PLBs survived in the control experiment. Loading solution was reported to be useful as a cryoprotectant in simple (two-step) freezing method (Sakai et al., 1991; Nishizawa et al., 1992). Exposing cells to high concentrations of cryoprotective additives is potentially injurious due to the phytotoxic effects of individual components or their combined osmotic effects on cell viability (Towill and Jarret, 1992; Sakai, 2000). Thus, strategies have been devised to reduce their toxicity which includes sequential loading and unloading treatments to avoid osmotic shock, and application at chilling temperatures (Day et al., 2008). In addition to their physical protective properties, cryoprotectants also impart additional protection against cryoinjury as they can stabilize proteins and membranes and act as antioxidants (Fuller, 2004). In this study, three biochemical assays - chlorophyll and total soluble protein contents, and peroxidase activity - were conducted after the cryopreservation procedure. The post-cryopreservation analyses were required to ascertain the response of PLBs to several kinds of stresses and thus determine the ability of the tissues to survive and regrow.

In this study, it was observed that the control PLBs possessed the highest total chlorophyll content as compared to the cryopreserved and non-cryopreserved PLBs. Furthermore, all PLBs subjected to cryopreservation in this study bleached and displayed symptoms of hyperhydricity. The results are in agreement with Bukhov et al. (2006), who found that

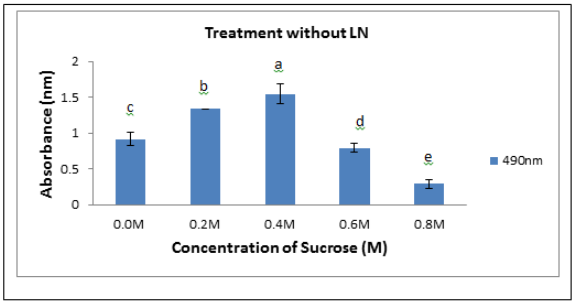

Fig 4. Effect of various concentrations of sucrose preculture on 3-4mm non-cryopreserved PLBs. Error bars represent the standard deviation of means.

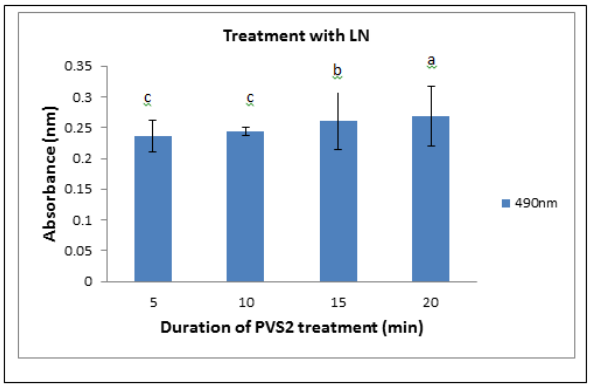

Fig 5. Effect of preculture of 3-4mm PLBs in 0.8M sucrose, followed by dehydration in PVS2 for 5, 10, 15 or 20 minutes, prior to cryopreservation. Error bars represent the standard deviation of means.

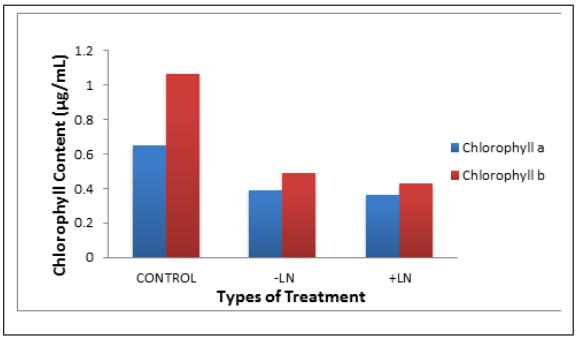

Fig 6. Chlorophyll *a* and chlorophyll *b* contents of the control, treated and cryopreserved PLBs after two weeks of recovery from their respective treatments.

cryopreserved *Bratonia*'s protocorms remained green for one month after thawing when placed in darkness but bleached within three to five days after their transfer to light. They proposed three possibilities, (i) the cryopreservation inhibited the activities of both photosystems I and II to a certain extent, (ii) the phototrophic cells in the protocorms of *Bratonia* retained the activities of both noncyclic electron flow and of photosystem I-dependent alternative electron transport pathways, indicating that the deep-freezing did not cause an immediate death of the protocorms and (iii) the cryostored and thawed protocorms experienced full inhibition of photosynthetic activity the day after thawing, causing progressively increased damage to the protocorms and

resulting in their death in the following culture cycles. The Bradford method was used to determine the protein content of PLBs of *Brassidium* Shooting Star (Bradford, 1976) as it is simple, fast and inexpensive (Nuria and Pilar, 2001). In this study, cryopreserved PLBs recorded the lowest total soluble protein content, while control PLBs produced the highest total soluble protein content. The normal metabolic and growth activities of plants decrease when subjected to any biotic or abiotic stress factors (Nuria and Pilar, 2001). Furthermore, there is a high possibility that the PLBs subjected to cryopreservation possessed low or no stress tolerance mechanisms, thus resulting in low total protein production. Thierry et al. (1999) reported that major proteins such as storage proteins are not accumulated excessively in cryopreserved and non-cryopreserved carrot somatic embryos during pretreatments involving sucrose or abscisic acid. It was also suggested that many boiling-stable proteins such as the late embryogenesis-abundant (LEA) protein, desiccation and cold acclimation-induced proteins were involved in the protection mechanisms for the preservation of protein and membrane structure, sequestration of ions, chaperone-like functions (Bray, 1993; Pelah et al., 1997; Bukhov et al., 2006), and resistance against freezing damage (Ausborn et al., 1994; Bukhov et al., 2006). The proteins are also involved in the acquisition of cryotolerance of somatic embryos, preventing occurrences such as crystallisation in cells and denaturation of membranes, proteins and nucleic acids, and the protection of in vitro lactate dehydrogenase enzyme from freeze-inactivation (Guy, 1990 ; Bukhov et al., 2006).

In this study, the highest peroxidase activity was obtained from cryopreserved PLBs, followed by non-cryopreserved PLBs, and control PLBs. The higher peroxidase activity observed in plants submitted to stress can indicate the ability of certain genotypes to degrade toxic substances, such as free radicals (peroxides) released under these conditions (Campa, 1991). There is a complex involvement of peroxidase in plants grown under stress (Gasper et al., 1985). Consequently, under the various stressed conditions (cryopreservation), PLBs could secrete higher amount of the peroxidase enzyme from cell walls as a defence mechanism.

The photosynthetic and antioxidant systems in plants are closely linked to one another in terms of their processes and functionality. The water cycle in chloroplasts is defined as a process in which excess photons are removed and active oxygen molecules are scavenged (Asada, 1999; Bukhov et al., 2006). The system relies on the structural integrity of chloroplasts, redox state and the control of the production of reactive oxygen species (ROS) through antioxidant protection and metabolic coupling, hence protecting the plant from excess photon energy and photo inhibition. Antioxidant enzymes, such as superoxide dismutase, that are inactivated by chilling, may cause photo inhibition of photosystem I.

Materials and Methods

Plant material and propagation of cultures

In vitro culture of protocorm-like bodies (PLBs) of Brassidium Shooting Star were initiated by aseptically culturing shoot tips of the plant in half strength semi-solid Murashige and Skoog (1962) media supplemented with 1mg/L BAP, 2% (w/v) sucrose and 2.75g/L Gelrite™. The resulting PLBs were grown at 25°C under 16 hours photoperiod. The PLBs were then subcultured for every 4 weeks in half strength liquid Murashige and Skoog (1962) media supplemented with 1mg/L BAP and 2% (w/v) sucrose.

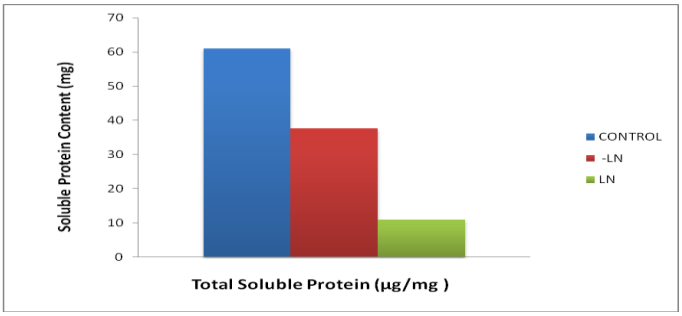

Fig 7. Total soluble protein contents of the control, treated and cryopreserved PLBs after two weeks of recovery from their respective treatments.

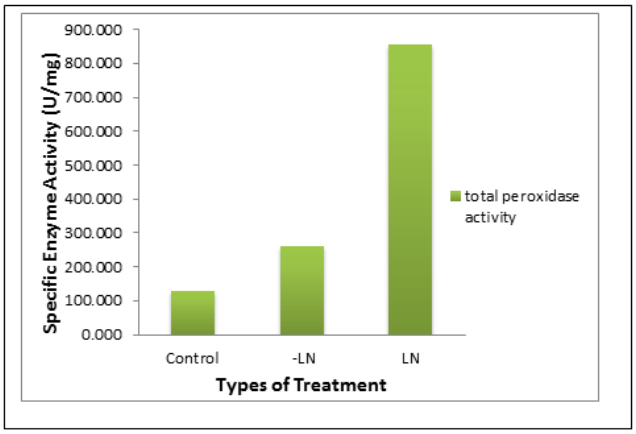

Fig 8. Peroxidase activity of control, treated and cryopreserved PLBs after two weeks of recovery from their respective treatments.

Media preparation

All media used in the experiment were supplemented with half-strength MS medium. The preculture media were supplemented with various concentrations of sucrose (0.0 [control], 0.2, 0.4, 0.5, 0.6, and 0.8M), while the loading solution contained 0.4M sucrose and 2.0M glycerol. The PVS2 consisted of 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) DMSO and 0.4M sucrose (Sakai et al., 1990), and the unloading solution contained 1.2M sucrose. The recovery medium consisted of semi solid half-strength MS, supplemented with 2% sucrose and 2.75g/L Gelrite™. The pH of all media was adjusted to 5.8 prior to autoclaving, with the sterilising conditions set to 121 °C / 250 °F for 30 minutes (STURDY SA-300VFA-F-A505, Sturdy Industrial Co. Ltd., Taiwan).

Preculture, Osmoprotection and Dehydration of PLBs of Brassidium Shooting Star

Four-week old PLBs were aseptically excised into two different sizes (1-2 and 3-4 mm), and cultured on medium supplemented with 0.5M sucrose to select the best PLB size for the cryopreservation treatment. For treatments involving various preculture concentrations, 3-4 mm PLBs were precultured for 24 hours on medium supplemented with 0.0 (control), 0.2, 0.4, 0.6, and 0.8M sucrose. Precultured PLBs were placed into 1.8ml plastic cryovials (Nalgene Nunc, United States of America [USA]) and immersed in 1.5ml loading solution for 20 minutes at 25°C (Matsumoto et al.,

1998). The loading solution was drained off from the cryovials and 1.5 ml of pre-chilled PVS2 (0^oC) was immediately added to the cryovials for 5, 10, 15 or 20 minutes prior to cryostorage. The cryovials, together with the samples, were then rapidly immersed into LN (MVE Lab 20, MVE Bio-Medical Division, Chart Industries, Inc., USA) for at least 1 hour (Panis et al., 1996; Leunufna and Keller, 2003). The loading solution was replaced with 1.5 ml unloading solution for 20 minutes in the control treatment. The control treatment involved PLBs that were not subjected to any of the steps mentioned above, while treated PLBs were those that were subjected to preculture, osmoprotection, dehydration and growth recovery steps, without undergoing the cryostorage and thawing steps. Cryopreserved PLBs were those that underwent each step of the cryopreservation experiment.

Thawing and Growth Recovery

Thawing was conducted for the cryopreserved PLBs in a water bath set at 40^oC for 90 seconds (Matsumoto et al., 1995; Panis et al., 1996; Leunufna and Keller, 2003). After thawing, the PVS2 solution was removed from the cryovials and the samples were immediately treated with 1.5ml unloading solution. The PLBs were then placed on recovery medium affixed with a piece of filter paper, followed by incubation in the dark at room temperature for a day. After one day, the PLBs were transferred to a new growth recovery medium and left under dark conditions again, for the next seven days. The PLBs were then gradually exposed to light for two weeks.

2,3,5-Triphenyltetrazolium chloride (TTC) Assay

In this study, the TTC assay was adapted from (Steponkus and Lanphear, 1967; Harding and Benson, 1995) with modifications. Each replicate of the experiment, containing 10 PLBs, was retrieved from the recovery medium and placed into universal bottles (Dimention; 31.42 cm^3). Two ml of the TTC buffer (0.6% [w/v] TTC, 0.05M KH_2PO_4, 0.05M $Na_2HPO_4.2H_2O$, 0.05% [v/v] Tween 80, pH 7.4) were pipetted into the bottles followed by incubation of PLBs in the solution in the dark, at 30°C, for 24 hours. After the incubation, the TTC buffer solution was discarded, and the PLBs were washed three times with 3.5ml distilled water. The formazan within the PLBs were then extracted with 7ml of 95% ethanol in a water bath set to 80°C for one hour. The formazan extracts were cooled to room temperature, topped up to 7ml with 95% ethanol and measured using a spectrophotometer (U-1900 UV-VIS Spectrophotometer 200V, 3J0-0003, Hitachi, Japan) at 490nm.

Biochemical analyses of the cryopreserved PLBs

Plants that experience chilling or freezing injuries must be able to regulate and protect the oxidative processes in both their photosynthetic and respiratory pathways (Benson and Bremner, 2004). Therefore, 3-4mm *Brassidium* PLBs subjected to the following treatment: preculture in 0.8M sucrose for 24 hours and dehydration for 20 minutes in PVS2 at 0^oC, were used to detect biochemical differences in both cryopreserved and non-cryopreserved *Brassidium* PLB samples.

Chlorophyll analysis

The chlorophyll content determination protocol was adapted from Harborne (Harborne, 1973).

Enzyme Extraction

One gram of PLBs derived from cryopreserved or non-cryopreserved PLB samples were ground in 3ml protein extraction buffer in an ice bath. The protein extraction buffer consisted of 0.1M Tris-hydrochloric acid and 1.0mM EDTA dissolved in double distilled water containing 0.1% mercaptoethanol, with the pH adjusted to 8. The extract was centrifuged at 12,000 rpm at 4^oC for 20 minutes. The supernatant was used as the sample to determine the total soluble protein content of the PLBs.

Total soluble protein

The Bradford method (Bradford, 1976) was used to determine the protein content of the PLBs.

Peroxidase activity

The peroxidase assay was adapted from Flocco and Giulietti (2003). The PLB samples, weighing 100mg, were immersed in 3ml of 100mM sodium phosphate buffer (pH 6). The mixture was centrifuged at 8000g (9176 rpm) for five minutes. The supernatant (10µl) was mixed with 3ml guaiacol reagent (0.35% guaiacol in 100mM KH_2PO_4, pH 7.4) and 10µl 30% H_2O_2. The mixture was then inverted three times, followed by immediate measurements of the supernatant at 470 nm every 30 seconds for three minutes.

Data analysis

The experiments consisted of three replicates, with each replicate containing 10 explants. The 10 PLBs were ultimately pooled in each replicate for each of the tests conducted above. Means were analysed through the Independent Samples' T-test in the selection of the best PLB size for cryostorage, with level of significance set at 0.05. Means in the other experiments were analysed through the one-way analysis of variance (ANOVA) and differentiated with Tukey's test, with the level of significance taken at 0.05.

Conclusion

The best results in the cryopreservation of the PLBs of *Brassidium* Shooting Star were obtained when the PLBs were precultured for 24 hours in half-strength MS semi-solid medium supplemented with 0.8M sucrose, followed by a 20-minute loading treatment at room temperature and a 20 minute dehydration treatment in PVS2 at 0^oC before immediate storage in liquid nitrogen. The highest total soluble protein and chlorophyll contents were found in control PLBs, while PLBs that were stored in liquid nitrogen produced the lowest values for both the tests. However, the peroxidase and specific enzyme activities were determined to be the highest in cryostored PLBs. Hence, the optimised method can be adapted as a cryopreservation procedure for PLBs of the orchid hybrid *Brassidium* Shooting Star.

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