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Full Length Research Paper

Preliminary analysis of cryopreservation of *Dendrobium* Bobby Messina orchid using an encapsulation-dehydration technique with Evans blue assay

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***In vitro* grown protocorm-like bodies (PLBs) of *Dendrobium* Bobby Messina hybrid were cryopreserved in liquid nitrogen (LN) at -196°C by an encapsulation-dehydration technique. PLBs (1 to 2 and 3 to 4 mm) were precultured in half strength semi-solid MS media supplemented with six different concentrations of sucrose (0.0, 0.2, 0.4, 0.6, 0.8 and 1.0 M). The PLBs were then encapsulated to form the beads in half-strength liquid MS media supplemented with different concentrations of sodium alginate (2.5, 3.0 and 3.5%). The beads were placed in 2 ml cryovials and plunged into LN for 24 h. The beads were then thawed in a 40°C water bath for 90 s and were placed in recovery media composed of half strength semi-solid MS media supplemented with 2% sucrose for four days under dark condition. After 12 days, the Evans blue dye assay was carried out to determine the viability of the PLBs. The highest viability was found in 1 to 2mm PLBs precultured in half strength semi-solid MS media supplemented with 1.0 M sucrose and encapsulated in 2.5% sodium alginate. Biochemical content analyses (chlorophyll, total soluble protein and peroxidase activities) were done to investigate the physiological responses of the PLBs after cryopreservation.**

Key words: Orchid, protocorm-like bodies, *Dendrobium* Bobby Messina, encapsulation-dehydration.

INTRODUCTION

The family Orchidaceae is the most popular group of ornamental plants in the world. Their unique characteristics and long flower lifespan contributes to the popularity of the flowers, hence being marketed both as plants and cut flowers (Goh, 1983). Recent published data indicated that orchids represent 8% of the global floriculture trade with *Dendrobium* hybrids being commercially desirable due to the high number of flowers per inflorescence and recurrent flowering of the plant (Martin and Madassery, 2006). *Dendrobium* Bobby Messina is a new orchid hybrid with high potential of becoming popular in the orchid industry. In cryopreservation of protocorm-like bodies of *Dendrobium* Bobby Messina by vitrification method, protocorm-like bodies (PLBs) with 3 to 4 mm size range showed better viability which is comparable to size range 1 to 2 mm for both cryopreserved and non-cryopreserved PLBs. The best preculture concentration used in pretreatment media

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Abbreviations: LN, Liquid nitrogen; **MS,** Murashige and Skoog medium; **PLB,** protocorm-like body; **TTC,** 2,3,5-triphenytetrazolium chloride.

was 0.6 M sucrose by using triphenyl tetrazoliumchloride (TTC) spectrophotometrical analysis (Antony et al., 2010).

Factors such as the green house effect and uncontrollable harvest make valuable tropical orchids to be extinct, due to extensive disturbances to their life cycle and natural habitat (Thammasiri, 2005). Therefore, the genetic resources of orchids should be preserved. There are two ways of preserving germplasm; *in situ* conservation maintains plant species in their own natural habitat and *ex situ* conservation maintains plants species under artificial condition by propagation, micro-propagation or *in vitro* culture (Hirano et al., 2005). However, the *ex situ* method is preferred for orchid species or hybrids since *in-situ* conservation requires large amount of field gene banks or botanical gardens. Therefore, effective *ex situ* conservation methods are needed to achieve long-term storage of orchid genetic resources. Thus, cryopreservation is the most reliable method in the *ex situ* conservation methods (Engelmann, 2000).

Cryopreservation is the storage of tissues in liquid nitrogen (-196°C) and it is considered as the best option for long-term storage of germplasm. At this temperature, all cellular divisions and metabolic processes of stored cells are halted (Engelmann, 2000) and biochemical processes are arrested (Niino and Sakai, 1992). Several methods have been developed to preserve plant germplasm such as vitrification, encapsulation-dehydration and encapsulation-vitrification (Sakai, 1997).

The encapsulation-dehydration technique of cryopreservation developed by Fabre and Dereuddre (1990) is based on the technology for the production of seeds. In this technique, the explants are encapsulated in beads, dehydrated and then cooled rapidly in liquid nitrogen (Sakai et al., 2000). This method is simple, inexpensive and the high genetic stability of cryopreserved material can be maintained (Kartha and Engelmann, 1994). It is easy to handle and tissues are directly immersed into liquid nitrogen without being exposed to toxic cryoprotectants. Thus, this method solely depends on the presence of sugar in pretreatment as its cryoprotectant.

The physiological status of the plant material is important for the success of plant cryopreservation (Engelmann et al., 2008). Samples are excised from actively growing mother plants, thus ensuring that they are composed of actively dividing meristematic cells (Engelmann, 1997; Escobar et al., 1997). Each PLB has two ends directly opposite to each other: the shoot region where the shoot appears and the root region where the root system is located. The new cells tend to propagate on the surface of the original PLB to form a clump of tissues. PLBs are used as the target tissues for cryopreservation since they are easily propagated *in vitro*, providing plenty of materials to work with and they were proven to be a reliable source of potentially regenerable tissues (Ishikawa et al., 1997).

Sodium alginate consists of alginic acid which turns to insoluble gel after contact with divalent metal ions, such as calcium (Kakita and Kamishima, 2008). Beads consisting of sodium alginate coating PLBs are held in the calcium chloride solution for 20 to 30 min to guarantee good polymerisation (Englemann et al., 2008). Several main characteristics of alginic acid that promotes its use for encapsulation are: (1) high viscosity solution and spin tolerance; (2) ability to form gels in the presence of certain divalent (or multivalent) cations, particularly calcium ions; (3) adhesive and biocompatible properties; and (4) ability to entrap other materials in the gel (Kakita and Kamishima, 2008). An alginate-gelled matrix surrounding the explants slows the process of desiccation and provides the mechanical support needed to protect the tissue during long-term storage (Sujatha et al., 2007).

The concentration of chlorophyll *a* and *b* can be determined by using chlorophyll assay (Harborne, 1973). Chlorophyll *a* is the main molecule which allows photosynthesis to occur as it is the main photosynthesis pigment of aerobic organisms such as plants, algae and cyanobacteria (Devesa-Rey et al., 2008). Besides, chlorophyll *b* occurs only in green algae and in plants, and its main function is to absorb light and transfer it to chlorophyll *a* (Devesa-Rey et al., 2008).

There are two major techniques to determine the viability of the plant cells; those that stain only the dead cells and those in which only the living cells are coloured, as the colour is a product of cell metabolic activity (Widholm, 1972). Methods of evaluating plant cell viability include using stains (Evans blue, bromophenol blue, methylene blue and phenosafranin) or assays, for example tetrazolium salts such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and triphenyl tetrazolium chloride (TTC) (Fernandez-Da and Menéndez-Yuffà, 2006).

Post-cryopreservation analyses can be applied to determine the viability and regrowth of cryopreserved tissues. Biochemical tests such as the chlorophyll analysis, total soluble protein analysis, acid phosphatase activity and peroxidase activity may be used to both evaluate the biochemical content in explants and detect new proteins produced by cryopreserved plants.

The aim of this study was to develop an optimised cryopreservation protocol for *Dendrobium* Bobby Messina by encapsulation-dehydration technique.

MATERIALS AND METHODS

In vitro cultures of PLBs of *Dendrobium* Bobby Mesinna were selected for cryopreservation in this study. Stock cultures were cultured in half strength Murashige and Skoog media (MS) (1962) supplemented with 1 mg/L benzylaminopurine (BAP), 2% sucrose and 2.75 g/L gelrite. The pH was adjusted to 5.8 prior to autoclaving. The PLBs were grown at 24°C under 16 h photoperiod (Philips TLD, 36 W) at 150 µmol m^{-2} s^{-1}. The PLBs were then subcultured for every 4 weeks (Figure 1).

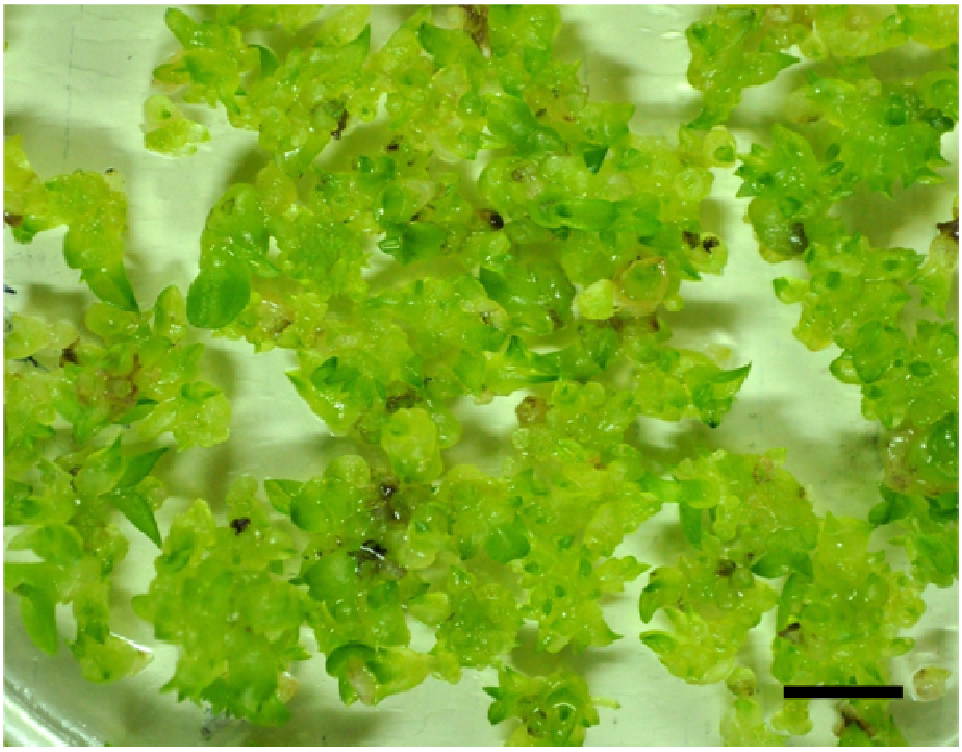

Figure 1. Stock culture of PLBs of *Dendrobium* Bobby Messina orchid hybrid. Bar = 1.0 cm.

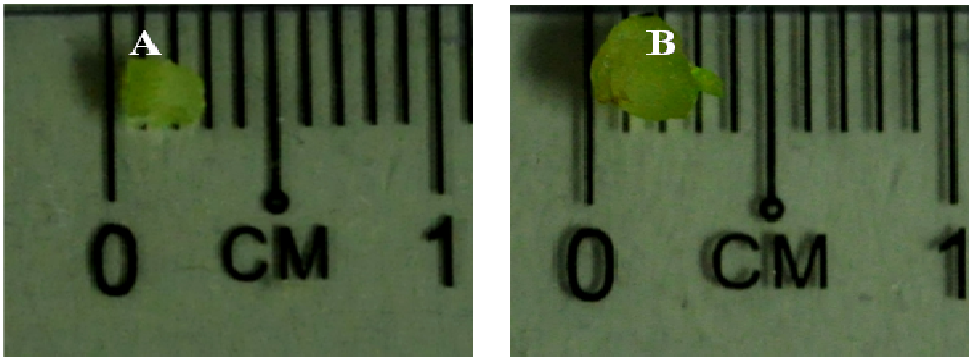

Figure 2. One to two millimetres PLB (A) and three to four millimetres PLB (B).

Preculture of different sizes of PLBs in various concentrations of sucrose

The entire protocol was conducted under the sterile air flow of the laminar flow cabinet. One(1) to Two(2) mm (Figure 2a) and 3 to 4 mm (Figure 2b) PLBs (10 PLBs each replicate) were precultured in half-strength semi-solid MS media supplemented with six different sucrose concentrations (0, 0.2, 0.4, 0.6, 0.8 and 1.0 M) for 24 h at 25 ± 2°C following 16-h photoperiod under cool white fluorescent lamps (Philips TLD, 36 W) at 150 µmol $m^{-2}s^{-1}$.

Alginate encapsulation in different concentrations of sodium alginate

The precultured PLBs were suspended in either 2.5, 3.0 or 3.5% sodium alginate supplemented with 0.4 M sucrose in calcium free half strength liquid MS media. Each PLB was aspirated with 100 µl of the alginate medium with a pipette fitted with a tip modified to a diameter of 5 mm, and the entire content was dropped into 0.1 M calcium chloride supplemented with 0.4 M sucrose in half strength liquid MS media (Figure 3).The beads were left to harden for 30 min.

Osmoprotection

After 30 min, the beads were collected and osmoprotected in half strength liquid MS media supplemented with 0.75 M sucrose on an orbital shaker (NB-101M2) at 120 rpm for 24 h at 25 ± 2°C following 16-h photoperiod under cool white fluorescent lamps (Philips TLD, 36 W) at 150 µmol $m^{-2}s^{-1}$.

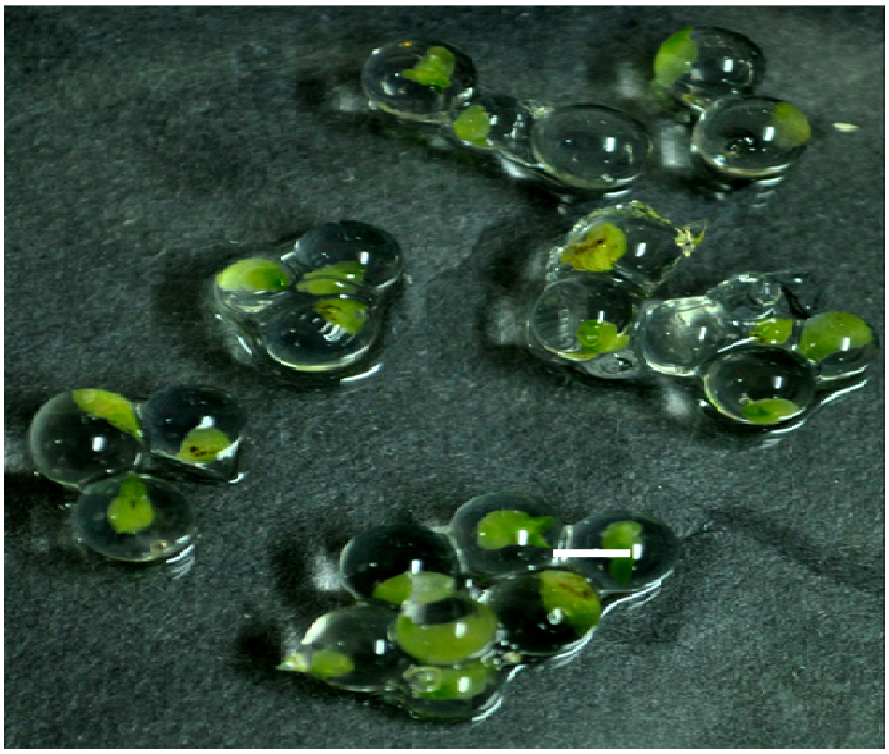

Figure 3. PLB encapsulation using sodium alginate and 0.1 M calcium chloride solution. Bar = 5 mm.

Dehydration using silica gel

Osmoprotected beads were removed from the liquid medium and were surfaced dried by blotting them on sterile filter paper. The beads (10 beads per replicate) were then dehydrated for 3 h on a piece of sterile filter paper fitted into a hermetically-sealed culture jar containing 50 g of silica gel presterilised at 103°C overnight.

Cryostorage and thawing

After dehydration, the beads were placed in 2 ml cryovials and were directly plunged into liquid nitrogen for 24 h. Cryopreserved PLBs were thawed by rapid warming in a water bath at 40 ± 2°C for 90 s.

Growth recovery

Beads were removed from the cryovials and cultured on recovery media consisting of half-strength semi-solid MS media supplemented with 2% sucrose. The cultures were kept in the dark for four days. The Petri dishes containing the encapsulated PLBs were then slowly acclimatised to light by covering them with a white cloth while being exposed to a light intensity of 150 µmol $m^{-2}s^{-1}$ to minimise light penetration. This was conducted for a total of four days. After that, the PLBs were exposed directly to light for four days before viability tests were conducted.

Evans blue dye technique

The viability of the cryopreserved protocorm-like bodies (PLBs) cells was measured using the Evans blue dye technique, since the dye stains dead cells and higher absorbance value indicates lower viability (Baker and Mock, 1994; Ikegawa et al., 1998; Heine-Dobbernack et al., 2008). PLBs were extracted out of their capsules and were transferred into 2 ml test tubes. Then, 1.5 ml Evans blue solution was added into the test tube, and the PLBs were incubated at room temperature for 15 min. The solution was subsequently removed and the PLBs were washed five times with distilled water until all traces of the dye were removed. The PLBs were later ground using micro pestle with 1.5 ml of 2% sodium dodecyl sulphate (SDS) solution. The mixture was transferred into Eppendorf tubes and the PLBs were centrifuged at 14,000 rpm for 15 min. The supernatant was used for the spectrophotometric analysis at 600 nm (Spectro 22 Digital Spectrophotometer, Labomed Inc.).

Chlorophyll analysis

The chlorophyll content was determination based on Harborne (1973) method.

Total soluble protein

The protein content of the PLB extract was determined using the Bradford (1976).

Peroxidase activity

The peroxidase activity of the PLB extract was determined using the Flocco and Giulietti (2003) method.

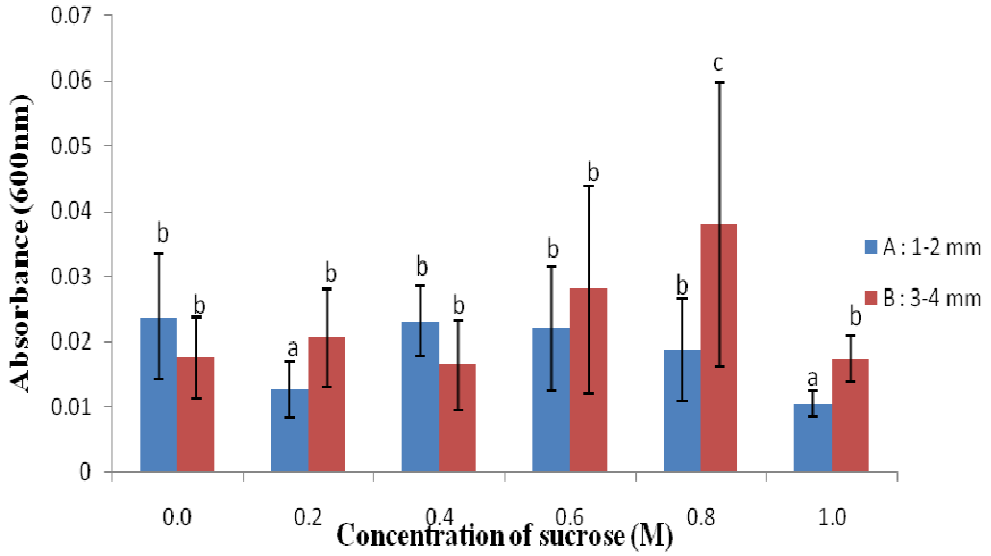

Figure 4. The effect of PLB size and preculture concentration on viability of cryopreserved PLBs. Results were analysed by one way ANOVA and means were compared by Tukey test. Vertical bars represent ± SD of means of six replicates.

Experimental design

Each experiment consisted of six replicates per treatment with 10 samples each.

RESULTS AND DISCUSSION

Optimisation of PLB size and preculture sucrose concentration

Preculture of the PLBs was necessary in order to minimise freezing injuries when plant tissues are cryopreserved. Generally, the explants are precultured with sucrose, sorbitol or mannitol for several hours or days after excision and before the encapsulation process (Engelmann et al., 2008). Sucrose and sorbitol are common osmotic reagents used in the preculture media, and they provide a valuable method for improving survival of cryopreserved shoot tips (Niino and Sakai, 1992; Yamada et al., 1991). Preculture with sucrose can reduce the water content in the cells and prevent ice crystallisation. The advantages of using sucrose in cryopreservation could be due to two effects (Steponkus et al., 1992). Firstly, sucrose provides an osmotic dehydration effect during treatment leading to reduced water content in the tissues (Tanaka et al., 2004). Secondly, sucrose molecules enter plant cells (Dumet et al., 1993) and replace the water content in the cells. Additionally, sugar also play an important role in the preservation of membrane integrity (Crowe et al., 1988) and protein structure during dehydration (Leslie et al., 1995). There is evidence that sucrose is able to enter the cells based on the histological observations of intracellular accumulation of starch during preculture (Gonzalez-Arnao et al., 1993).

In this study, 1 to 2 mm PLBs precultured with half strength semi-solid MS media supplemented with 1.0 M sucrose showed the highest viability rate (lowest reading). However, 1 to 2 mm PLBs precultured with half strength semi-solid MS media supplemented with 0.2 M of sucrose also showed similar viability (Figure 4). Therefore, 1 to 2 mm PLBs were precultured with half strength semi-solid MS media supplemented with 0.2 and 1.0 M sucrose for the subsequent experiments. Based on Evans blue assay, lower absorbance values indicate higher viabilities of the explants. However, in cryopreservation of protocorm-like bodies of *Dendrobium* Bobby Messina by vitrification method, PLBs with 3 to 4 mm size range showed better viability compared to size range 1 to 2 mm for both cryopreserved and non-cryopreserved PLBs. The best preculture concentration used in the pretreatment media was 0.6 M sucrose by using triphenyl tetrazoliumchloride (TTC) spectrophotometrical analysis (Antony et al., 2010). These differences in the results may be due to different cryopreservation method that was applied and due to different viability test that was used.

Optimisation of sodium alginate concentration

In this study, PLBs precultured in half strength semi-solid MS media supplemented with 0.2 and 1.0 M sucrose and encapsulated with 2.5, 3.0 and 3.5% sodium alginate showed no significant difference in the viability (Figure 5). Therefore, in this study, PLBs precultured in half strength semi-solid MS media supplemented with 1.0 M sucrose and encapsulated with 2.5% sodium alginate showed the

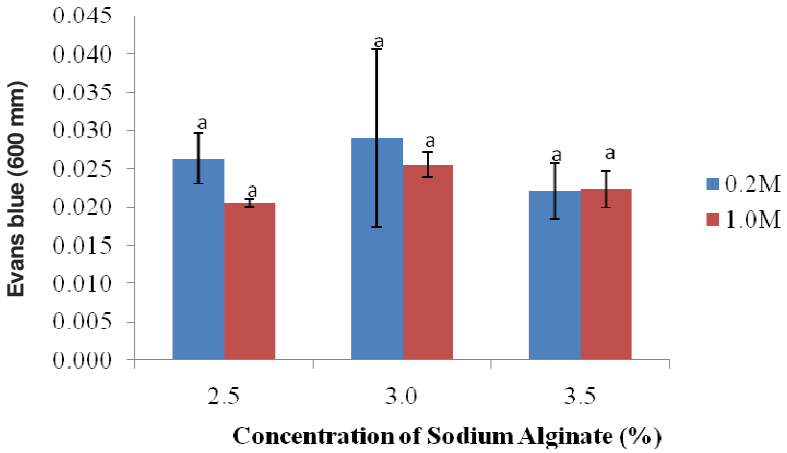

Figure 5. The effect of different sodium alginate concentrations on viability of cryopreserved PLBs. Results were analysed by one way ANOVA and means were compared by Tukey test. Vertical bars represent ± SD of means of six replicates.

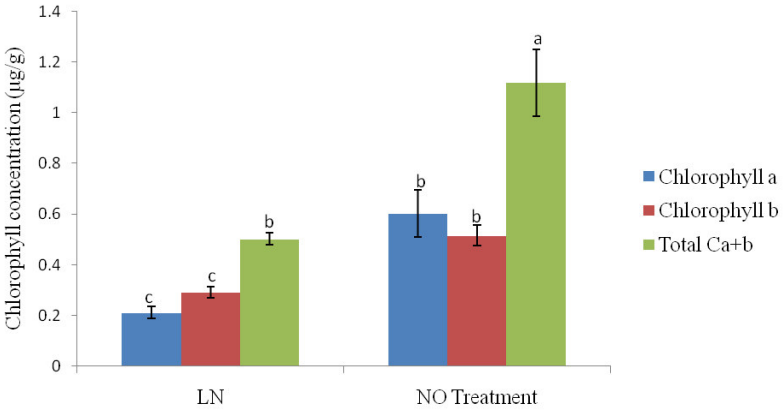

Figure 6. Chlorophyll content (mg/ml) in different samples applied in the encapsulation-dehydration technique. Untreated PLBs were obtained from original *in vitro* mother plants. Results were analysed by one way ANOVA and means were compared by Tukey test. Vertical bars represent ± SD of means of six replicates.

highest viability (Figure 5) and was chosen for the subsequent experiments. The 2.5% sodium alginate solution coated the PLBs very effectively and was the easiest to drop into the calcium chloride solution. The bead structure, although soft, held the PLBs in place and provided enough resistance from external mechanical pressure.

Chlorophyll analysis of the cryopreserved PLBs

In this study, the chlorophyll content (chlorophyll a, chlorophyll b and total chlorophyll content) was found to be higher in the non-treated PLBs as compared to cryopreserved PLBs (Figure 6). This showed that the chlorophyll content was reduced when the PLBs were treated in the presence of liquid nitrogen (LN).

Total soluble protein determination

If a plant is subjected to any biotic or abiotic stress factors, the first observed response will be a decrease in its normal metabolic activities, with a consequent reduction of growth. When this mechanism occurs, the protein synthesis is one of the most negatively affected anabolic process (Bohnert and Jensen, 1996), together with photosynthesis, transport of metabolites and uptake and translocation of ions (Hsiao, 1973; Lichtenthaler, 1996). In response to unfavourable conditions (for

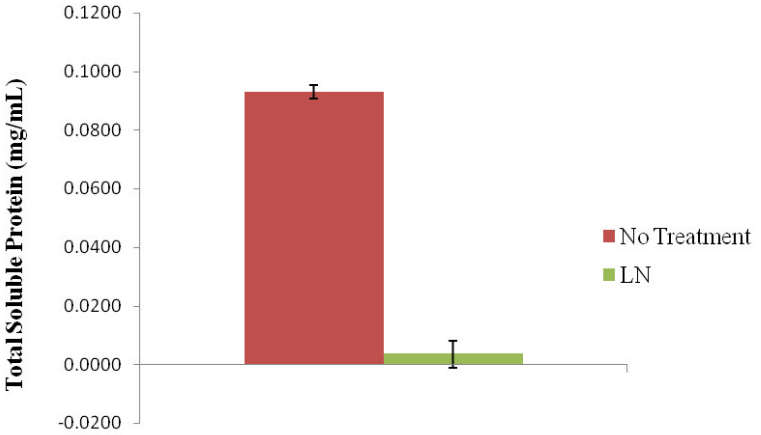

Figure 7. The effect of total soluble protein content in different treatments applied in the encapsulation-dehydration technique. Untreated PLBs were obtained from the original *in vitro* mother plants. Based on the independent sample t test, P-value = 0.000. Vertical bars represent ± SD of means of six replicates.

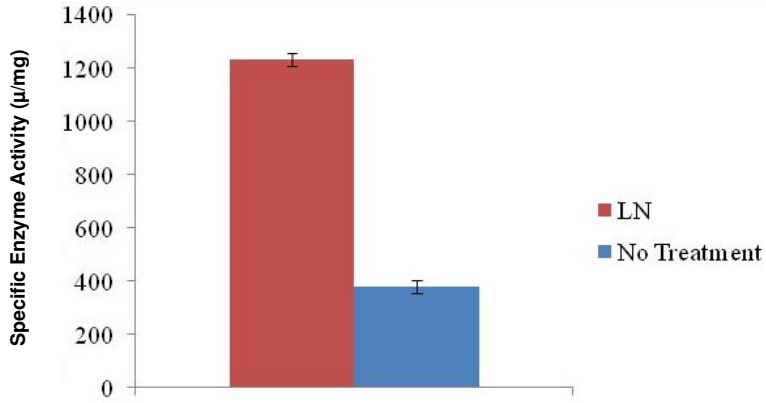

Figure 8. Specific peroxidase activities (U/mg) in different treatments applied using the encapsulation-dehydration technique. Untreated PLBs were obtained from the original *in vitro* mother plants. Based on Independent sample t test, P-value = 0.000. Vertical bars represent ± SD of means of 6 replicates.

example, -196°C of LN), most plants will activate their stress coping mechanisms such as activation of repair processes and long-term metabolic and morphological adaptations (Lichtenthaler, 1996). The mechanisms include *de novo* synthesis of protein with specific adaptive functions (for example, stress protein), osmotic adjustment and antioxidative defense. All these adaptive responses will carry modifications in the levels and activities of many enzymes of multiple metabolic pathways, which in return will affect the total protein levels of the plants (Bonjoch and Tamayo, 2001).

In this study, the untreated PLBs showed the highest value of total soluble proteins. The lowest value of total soluble proteins was found in PLBs stored in LN (Figure 7). The proteins levels of cryostored PLBs were reduced due to the stress caused by exposure to liquid nitrogen.

Peroxidase activity and specific enzyme activity

High peroxidase activity indicates the ability of certain plant genotypes to degrade toxic substances such as free radicals (peroxides) released under stress conditions (Campa, 1991). This indicates that there is a complex involvement of peroxidase activity in plants grown under stress condition (Gaspar et al., 1985).

In this study, peroxidase activity was highest in PLBs that were cryostored (Figure 8). Therefore, the PLBs

secreted a higher amount of peroxidase enzyme under stressed conditions.

Conclusions

In this study, the best results were obtained when 1 to 2 mm PLBs *Dendrobium* Bobby Messina were precultured in half-strength semi-solid MS medium supplemented with 1.0 M sucrose, and encapsulated with 2.5% sodium alginate. However, alterations in the chlorophyll content and total soluble proteins in PLBs occurred after they were cryopreserved. In addition, the highest total and specific peroxidase activities were reported in cryostored PLBs. Thus, further improvements and studies should be conducted in the cryopreservation of *Dendrobium* Bobby Messina in order to achieve higher survival rate of PLBs after cryostorage.

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