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Full Length Research Paper

Preliminary investigation of cryopreservation by encapsulation-dehydration technique on *Brassidium* Shooting Star orchid hybrid

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Protocorm-like bodies (PLBs) of *Brassidium* Shooting Star, a new commercial ornamental orchid hybrid, were cryopreserved by an encapsulation-dehydration technique. The effects of PLB size, various sucrose concentrations in preculture media and sodium alginate concentration for encapsulation were the main parameters assessed. Four-week old PLBs (1 to 2 and 3 to 4 mm) were precultured in half strength semi-solid Murashige and Skoog (MS) media supplemented with six different sucrose concentrations (0, 0.2, 0.4, 0.6, 0.8 and 1.0 M) for 24 h, followed by encapsulation in 2.5, 3.0 or 3.5% sodium alginate, with 0.1 M calcium chloride been used as the hardening agent. The beads formed were then osmoprotected in half-strength liquid MS media supplemented with 0.75 M sucrose and dehydrated for three hours in 50 g heat-sterilized silica gel before cryostorage in sterile cryovials. The beads were thawed in a 40 ± 2°C water bath and then directly placed in recovery media for two weeks under tissue culture conditions. After two weeks of recovery, the survival rates of the encapsulated PLBs were evaluated by the 2,3,5-triphenyltetrazolium chloride (TTC) assay. The best conditions for the encapsulation-dehydration of *Brassidium* Shooting Star were discovered to be the preculture of 3 to 4mm PLB in half strength semi-solid MS media supplemented with 0.8 M sucrose, followed by encapsulation in 3.5% sodium alginate. Further biochemical analysis (chlorophyll, total soluble protein and peroxidases activities) were conducted to investigate the physiological responses of the PLBs after cryopreservation.

Key words: Encapsulation-dehydration, cryopreservation, *Brassidium* Shooting Star, protocorm-like bodies.

INTRODUCTION

Orchids belong to the family Orchidaceae, the largest flowering plants (Angiospermae) group, and contain approximately 25,000 to 30,000 listed orchid species (Nikishina et al., 2001). Their unique floral characteristics make most orchids species distinct from other flowering plant. Orchids symbolizes an aristocracy in floriculture and horticulture (Chan et al., 2007), and most of the species are grown widely for production of commercial cut flowers and potted plants (Huang et al., 2009).

The International Union for Conservation of Nature and Natural Resources (IUCN) listed almost all orchid species as endangered in the Red Data Book. In Russia, several dozens out of about 150 species of wild orchids were recorded as nearly extinct (Nikishina et al., 2001). Orchids are at the edge of extinction due to several factors such as overexploitation, habitat loss and fragmentation, climate change impacts, altered abiotic conditions (for example, soil and hydrology), collapse in ecological links (for example, pollinators and mycorrhiza), weeds and introduced pests and diseases (Swarts and Dixon, 2000). Consequently, specific conservation strate-

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Abbreviations: MS, Murashige and Skoog (1962) medium; **2,4-D,** 2,4-dichlorophenoxyacetic acid; **IAA,** indole-3-acetic acid; **PGR,** plant growth regulator, **PLBs,** protocorm-like bodies.

gies should be designed to protect and preserve orchid populations and individuals as precious resources of heritable variation for future evolution.

In 1979, cryopreservation of shoot meristems, seeds and cells *in vitro* was proposed as a method to be used in conjunction with conventional methods of plant conservation (Nikishina et al., 2001). Cryopreservation involves the storage of living plant cells, tissues or organs at ultra-low temperatures (González-Benito et al., 1997). Cryopreservation is advocated as an ideal method for long-term storage of germplasm as all cellular divisions, enzymatic and metabolic processes of stored cells are halted at the low temperature used. Thus, plant materials can be stored with low risk of physiological and genetic alterations for an indefinite period of time (Wang et al., 2002). Several techniques such as vitrification, encapsulation-dehydration and encapsulation-vitrification, have been developed for conservation of endangered orchid species (Sakai et al., 2008).

Success in cryopreservation experiments depend on the physiological and developmental state of the target tissues. The plant material should be young and meristematic in origin, and the cells should contain a small number of vacuoles with minimal water contents and exhibit dense cytoplasm with high nucleo-cytoplasmic balance. The excised samples should be at a suitable growth stage to allow desiccation and freezing tolerance, with the ability to regenerate vigorous plantlets after cryogenic storage (Normah and Makeen, 2008).

Studies on recalcitrant and intermediate species had shown that the excised embryos and embryonic axes, including protocorms, have high potential in tolerating desiccation and cryoexposure than whole seeds (Bajaj, 1984; Radhamani and Chandel, 1992; Normah et al., 1994; Makeen et al., 2005; Normah and Makeen, 2008). Protocorm is a term created by the French botanist Bernard to indicate a developmental stage in the orchid embryo. Protocorms that were sectioned into several parts and transferred into new medium formed new protocorm clumps (Morel, 1963, 1974). 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 protocorms and protocorm-like bodies (PLBs), which are structurally and functionally similar to protocorms (Cherevchenko and Kushnir, 1986; Bukhov et al., 2006).

Brassidium Shooting Star is an orchid hybrid produced from a cross between the *Brassia* and *Oncidium* (*Oncidium wentworthianum* x *Brassia arcuigera* x *Brassia gireoudiana*) genus. Its unique spider-like flower structure led to its popularity in the floriculture business and its marketability all over the world. The objectives of this study were to determine the best PLB size, the best sucrose concentrations for preculture and the best alginate concentration for encapsulation and dehydration of PLBs of *Brassidium* Shooting Star, and also to compare the various biochemical activities of the cryopre-served and non-cryopreserved PLBs.

MATERIALS AND METHODS

Plant material

Protocorm-like bodie s (PLBs) o f *Brassidium* Shooting Star were used as the starting material to initiate PLB multiplication and propagation for this study. The cultures were subcultured once every four weeks, in half-strength semi-solid Murashige and Skoog (MS) medium supplemented with 2% sucrose, 2.75 gl^{-1} Gelrite™ and 1 $mgml^{-1}$ benzylaminopurine (BAP). The cultures were incubated under plant tissue culture room conditions (25 ± 2°C, 16 h photoperiod under cool white fluorescent lamps (Philips TLD, 36 W, 150 $\mu mol.m^{-2}.s^{-1)}$.

Media preparation

All media used in the experiment were supplemented with half-strength MS (Murashige and Skoog, 1962) medium components. The preculture media were supplemented with various concentrations of sucrose [0.0 (control), 0.2, 0.4, 0.5, 0.6 and 0.8 M], while the sodium alginate solution contained 2.5, 3.0 or 3.5% (w/v) sodium alginate and 0.4 M sucrose. The calcium chloride solution contained 0.1 M calcium chloride and 0.4 M sucrose, and the osmo-protection medium was supplemented with 0.75 M sucrose. The recovery medium consisted of pour plates of half-strength MS components, supplemented with 2% sucrose and 2.75 gl^{-1} gelrite. The pH of all media was adjusted to 5.8 prior to autoclaving.

Preculture

Four-week old PLBs were aseptically excised into two different sizes (1 to 2 and 3 to 4 mm) guided by a graph paper, and placed on medium supplemented with 0.5 M sucrose in order to select the best PLB size in the cryopreservation experiment. For experiments involving various preculture concentrations, 3 to 4 mm PLBs were precultured for 24 h on medium supplemented with the following concentrations of sucrose: 0.0 (control), 0.2, 0.4, 0.6 and 0.8 M, for 24 h. The preculture was conducted under plant tissue culture room conditions.

Encapsulation-dehydration

The precultured PLBs were then suspended in the sodium alginate medium. A single PLB was aspirated with 50 µl of the medium using a sterile micropipette fitted with a modified 1 ml tip (diameter = 4 mm) and dropped into the calcium chloride solution. The beads were allowed to harden in the solution for 30 min, with occasional agitation. The beads were collected and osmoprotected in the liquid osmoprotection medium on an orbital shaker set at 120 rpm for 24 h under plant tissue culture room conditions. Beads were then removed from the liquid medium and surface-dried by blotting them on sterile filter paper. Subsequently, the beads were dehydrated in hermetically-sealed culture jars containing 50 g of oven-sterilized silica gel (103°C) for four hours to a final water content of 19.9%, in the laminar air flow cabinet. The dehydrated beads were placed in 2 ml cryovials and immediately immersed in liquid nitrogen (-196°C) for 24 h. The beads were thawed in a 40°C water bath for 90 s, and cultured in the growth recovery medium. The cryostorage and thawing steps were skipped for the control experiments, and the dehydrated beads were immediately placed in the growth recovery medium. Both cryopreserved and non-cryopreserved beads were then incubated at 25°C in complete darkness for four days, under dim light (95 lux) for four days, and under 16 h photoperiod for six

days. The survival and biochemical tests were conducted after two weeks of growth recovery.

Determination of PLB survival through the 2,3,5-triphenyltetrazolium chloride (TTC) assay

The ability of plant mitochondria in living plant cells to reduce tetrazolium salts to easily measurable red formazan has made the TTC test a reliable and frequently used assay for cellular viability. The best method of assessing viability is through growth of the cryopreserved tissues into grown plants. However, some protocols require that the viability status of the target tissue should be known as soon as possible if the material is living after cryopreservation, because in many cases, regrowth is very slow (Normah and Makeen, 2008). This test, although destructive to the selected tissues, is qualitative for large tissues and organs when visualised under a microscope (Pellett and Heleba, 1998) or analyzed with a spectrophotometer (Harding and Benson, 1995), and is often used for embryos and embryonic axes (Normah and Makeen, 2008) for speedy assessment of the propagule's viability after cryopreservation as intensely coloured explants or extracts indicate higher levels of survival of the target explant. In this study, the TTC assay was adapted from Steponkus and Lanphear (1967), and Harding and Benson (1995) with modifications. Each replicate of the experiment was retrieved from the recovery medium and placed into universal bottles. Two milliliter of the TTC buffer [0.6% (w/v) TTC, 0.05 M KH_2PO_4, 0.05 M $Na_2HPO_4.2H_2O$, 0.05% (v/v) Tween 80, pH 7.4] were pipetted into the bottles. The PLBs were left to incubate in the solution in the dark, at 30°C, for 24 h. After the incubation, the TTC buffer solution was discarded, and the PLBs were washed thrice with 3.5 ml distilled water. The formazan within the PLBs were then extracted with 7 ml 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 7 ml with 95% ethanol and measured using a spectrophotometer (Hitachi U-1900) set at 490 and 530 nm.

Biochemical analysis

Chlorophyll content

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

Enyme extraction

The control (untreated), treated (-LN) and cryopreserved (+LN) PLBs were ground with protein extraction buffer with the ratio of PLB to buffer at 1:3 using a mortar and pestle placed in an ice bath. The protein extraction buffer consisted of 0.1 M tris hydrochloric acid, 1.0 mM EDTA and 0.1% mercaptoethanol dissolved in autoclaved distilled water. The pH of the buffer was adjusted to 8 with 0.1 M NaOH. The extract was then centrifuged at 12,000 rpm at 4°C for 20 min. The supernatant was used as the samples for the following experiments:

Total soluble protein content: The protein content determination of the PLB extracts was based on the Bradford (1976) method.

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

Statistical analysis

The experiments consisted of three replicates containing 10 explants. Means were analyzed 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 analyzed through the one-way analysis of variance (ANOVA) and differentiated with Tukey's test, with the level of significance taken at 0.05.

RESULTS AND DISCUSSION

Effect of PLB size and sucrose concentration

In the present study, 3 to 4 mm PLBs recorded a higher survival rate when compared to 1 to 2 mm PLBs (Figure 1). Large-sized explants had been observed to have more disadvantages in cryopreservation experiments when compared to small-sized explants. This is due to the presence of highly-vacuolated and hydrated cells in the former, which increases the risk of ice crystallization in the tissue. Large explants are also less efficient in heat dissipation during cooling as well as thawing (Panis et al., 2005). On the other hand, small explants often suffer from mechanical damage (Panis et al., 2005), hence causing difficulties in their handling. In this study, the 3 to 4 mm PLBs were selected for further experimentation.

Preculture with the presence of sugar is a crucial step for successful cryostorage of tissues in plant cryopreservation (Yin and Hong, 2009). Cryopreserved PLBs displayed increasing survival rates with increasing sucrose concentrations in the pretreatment medium (Figure 1). However, the survival percentage decreased when the PLBs were precultured in 1.0 M prior to cryopreservation. This could be attributed to the fact that high sucrose concentrations retard PLB growth, with tissue blackening seen as one of the symptoms of cellular death (Panis et al., 1996). In this experiment, the highest survival rate was obtained when 3 to 4 mm PLBs were precultured in medium containing 0.8 M sucrose prior to cryopreservation. It had been reported that sucrose concentrations ranging from 0.75 to 1.0 M are most frequently used for preculture in cryopreservation experiments (Yin and Hong, 2009). Sucrose preculture induces sugar accumulation, reduces water content and prevents ice crystallization in cells. It was 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 (Steponkus et al., 1992).

Effect of sodium alginate concentration

In this experiment, the highest survival of PLBs was re-

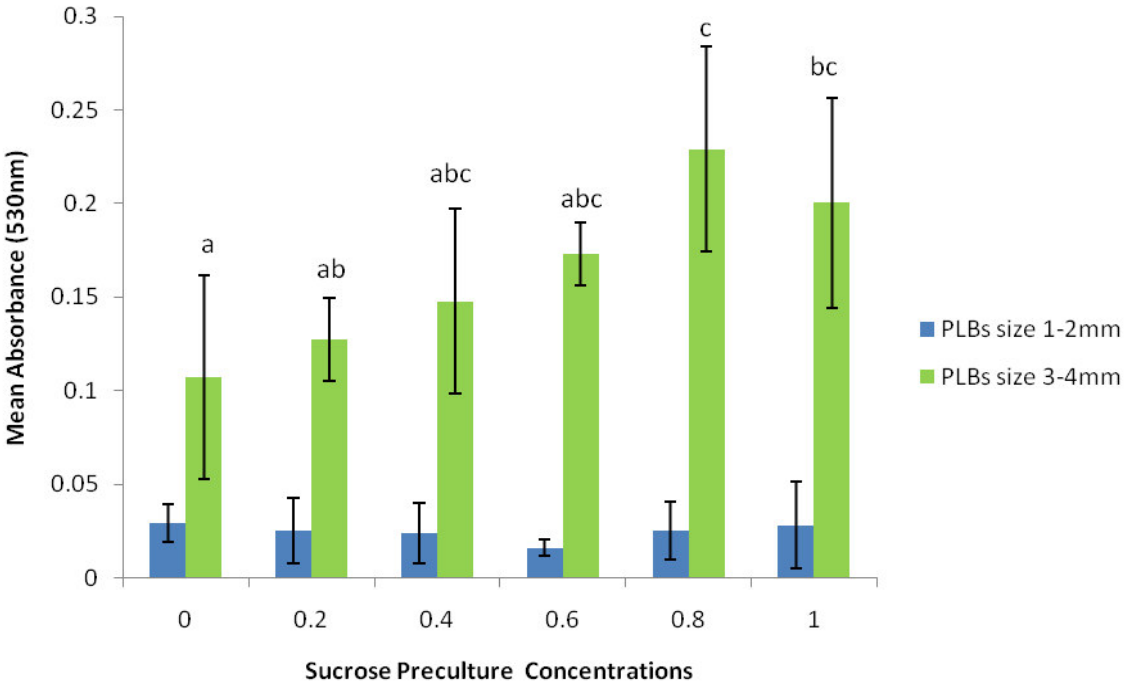

Figure 1. Effect of PLBs size and sucrose concentrations on survival after cryopreservation. The error bars represent the standard deviation of means of 3 replicates.

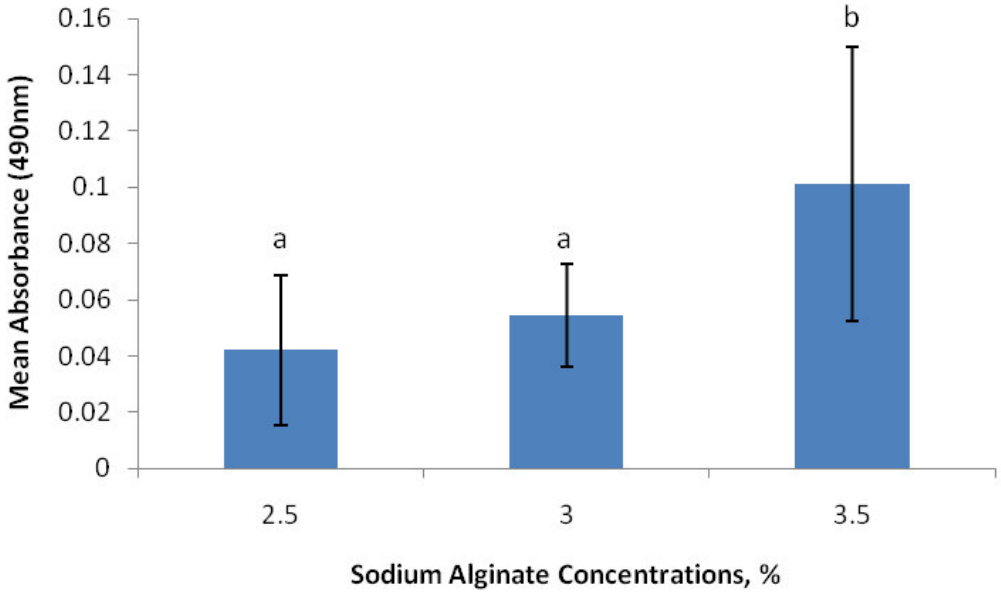

Figure 2. Effect of different sodium alginate concentrations on survival of cryopreserved PLBs. The error bars represent the standard deviation of means of 3 replicates.

corded when they were encapsulated using 3.5% sodium alginate (Figure 2). In general, 3% sodium alginate was found to be the optimal concentration to be used in bead preparation for explants. Firm and isodiametric beads were formed by complexing 3% sodium alginate with 0.1M $CaCl_2$ (Ballester et al., 1997). Sodium alginate, when prepared as a 5% (w/v) solution, produced the highest survival in the study of encapsulated *Camellia* shoot tips subjected to cold-storage (Ballester et al., 1997). It was also recommended that suitable combinations of nutrients, growth regulators and protectants were added to alginate beads to produce better growth yields

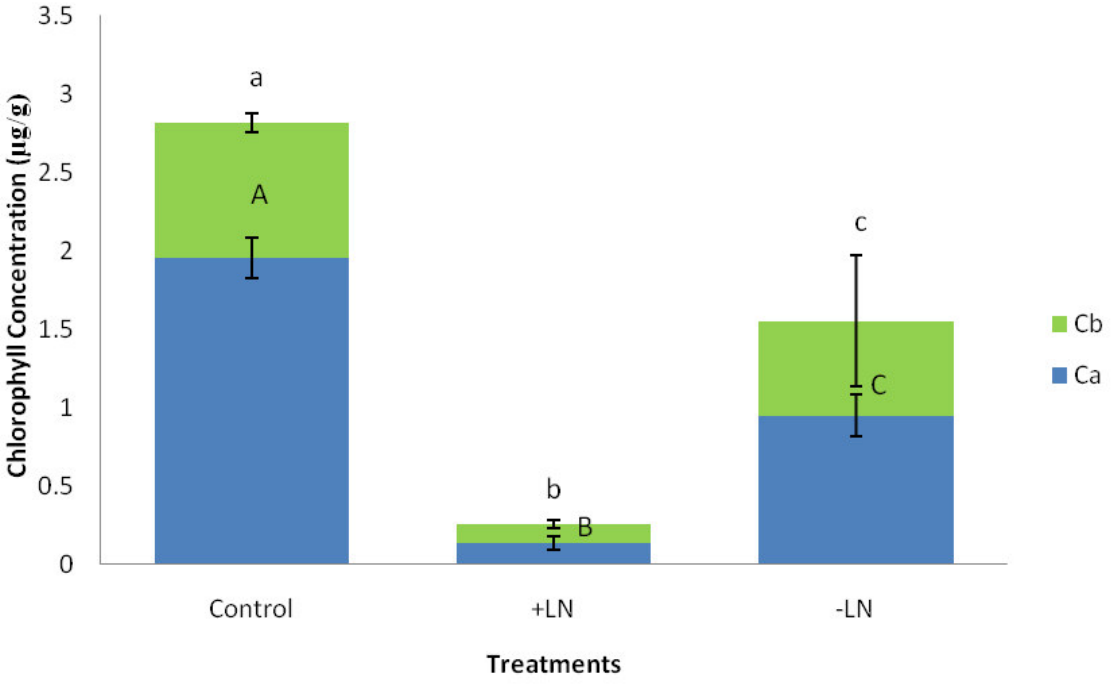

Figure 3. Effect of various treatments on the chlorophyll contents of cryopreserved, treated and untreated PLBs in the encapsulation-dehydration experiments. The error bars represent the standard deviation of means of 3 replicates.

in explants (Verma et al., 2010).

Chlorophyll content determination

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). The total chlorophyll *a* (C_a) contents of PLBs in all treatments were significantly ($p < 0.05$) higher than the chlorophyll *b* (C_b) contents (Figure 3). The highest total chlorophyll content was detected in the control (untreated) PLBs, amounting to 2.817 µg/g, while the lowest total chlorophyll content was detected in cryopreserved PLBs (0.256 µg/g).

There are two kinds of chlorophyll molecules in green plants: chlorophyll *a* and *b*. Plants are observed as green in white light as the chlorophyll absorbs energy from the blue- and red-coloured wavelengths of the light spectrum. In higher plants, most of the fluorescence comes from the chlorophyll *a* molecules, which are allied to the photosystem II at physiological temperatures (Reigosa and Weiss, 2003). Chlorophyll *b* amounts to about one-third of the chlorophyll *a* content in plants except in red algae and cyanobacteria (González, 2003).

Environmental stresses also cause pigment-level alterations to photosystem II. Heat, photo-inhibition and freezing can damage the thylakoids. Kautsky and Hirsch (1931) described the typical transient fluorescence phenomenon in the changing of illumination levels, which is also known as Kautsky-effect (Reigosa and Weiss, 2003). When light is shed upon a plant at a constant intensity, the emission of fluorescence is at a steady-state. However, a typical transient fluorescence phenomenon occurs when a dark-adapted plant (kept for several minutes in darkness) is suddenly exposed to bright light. It was also suggested that the chlorophyll *a* fluorescence emission exhibits a rapid increase to a maximum, followed by a slow decline to a steady level after a few minutes (Reigosa and Weiss, 2003). In this experiment, it was observed that cryopreserved PLBs maintained their green colour when stored in the dark, but bleached within two days of light exposure. This was not observed in uncryopreserved PLBs, which maintained their original growth capacity. Bukhov et al. (2006) concluded that the cryostorage of *Bratonia* protocorms caused disorders in the non-cyclic and cyclic electron transport system between photosystems I and II, most probably as a result of the disruption in the functional connection of electron carriers between the plastoquinone pool and the photosystem I reaction center.

Total soluble protein determination

The highest total soluble protein content was detected in PLBs subjected to the control experiment (13.222 µg/mg, Figure 4), while the lowest protein content was found in

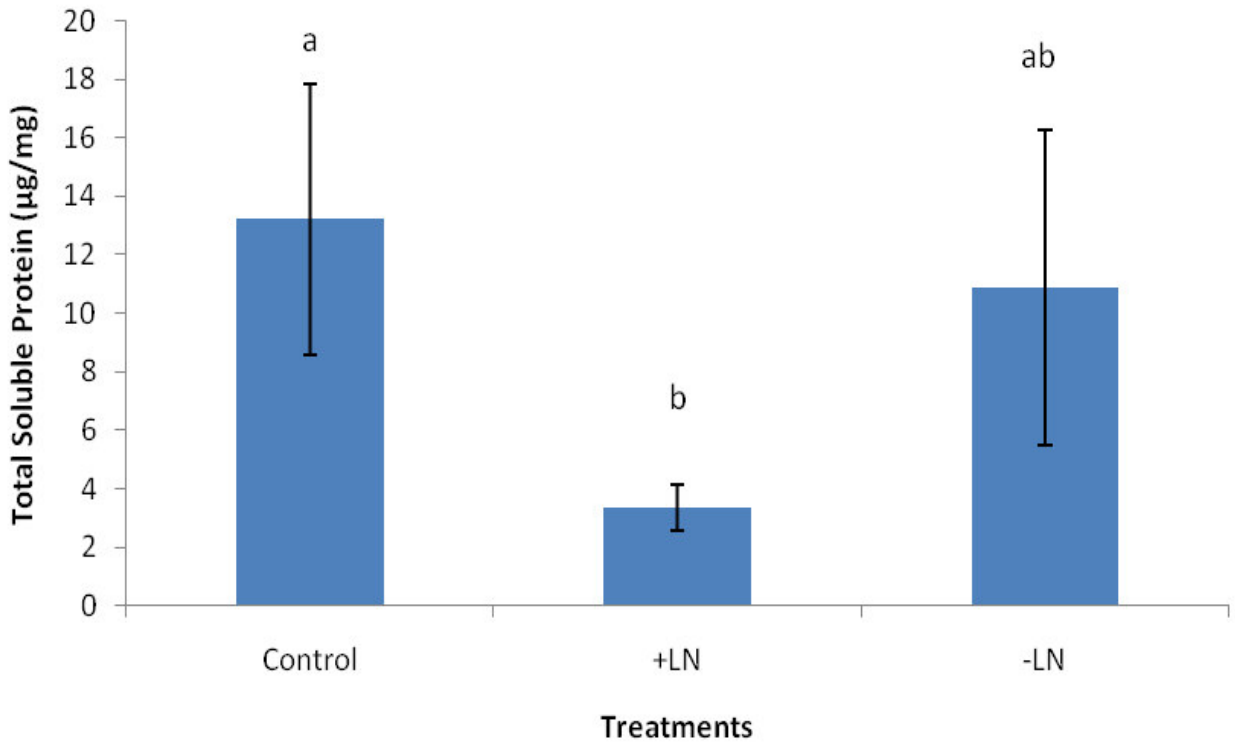

Figure 4. Effect of different treatments in encapsulation-dehydration on total soluble protein content of control, treated and cryopreserved PLBs. The error bars represent the standard deviation of means of 3 replicates.

cryopreserved PLBs (3.346 µg/mg, Figure 4). This could be attributed to the stress imposed on the PLBs by the cryopreservation treatments, causing a reduction in both PLB metabolism and growth. Protein synthesis has been found to be one of the most negatively affected anabolic processes together with photosynthesis, transport of metabolites, uptake and translocation of ions when plants were treated under stress conditions (Bonjoch and Tamayo, 2003). Thierry et al. (1999) discovered that major proteins, such as storage proteins, are not accumulated excessively in carrot somatic embryos during pretreatments involving sucrose or absisic acid, as observed in their 2-D polyacryamide gel electrophoresis (2-D PAGE) of total proteins found in cryopreserved and non-cryopreserved embryos.

Peroxidase activities

Cryopreserved PLBs of *Brassidium* Shooting Star recorded the highest values in both total peroxidase (37.541, Figure 5) and specific peroxidase (11.945 U/mg, Figure 6) activities. The total peroxidase activities for uncryopreserved PLBs and untreated PLBs were not significantly different from each other at 21.096 and 20.930, respectively (Figure 5). Similarly, the specific peroxidase activities for untreated PLBs and uncryopreserved PLBs were not significantly different from each other at 1.745 and 2.406 U/mg, respectively (Figure 6).

Typical plant peroxidases are heme-containing enzymes that catalyze the oxidation of a diverse group of organic compounds (Syros et al., 2004). Peroxidases are reported to be found in the vacuole (Yamauchi et al., 2004), and are involved in various metabolic processes such as auxin catabolism and the formation of isodi-Tyr bridges in the cross-linking of cell wall proteins (cell wall rigidification and lignification), which are participants in growth inhibition (Gaspar et al., 1985), removal of H_2O_2, oxidation of toxic reductants, defensive responses to wounding, defense against pathogen or insect attack, and some respiratory processes (Gulen and Eris, 2004). Alterations in peroxidase activity and peroxidase isoform patterns have been projected as biochemical markers of successive rooting phases (Syros et al., 2004). Chlorophyll-degrading peroxidase was reported to be present in the chloroplast and that the enhancement of its activity could be involved in degreening with senescence (Yamauchi et al., 2004).

Conclusions

The best conditions for the encapsulation-dehydration of *Brassidium* Shooting Star is the preculture of 3 to 4 mm PLBs in half-strength semi-solid MS medium supplemented with 0.8 M sucrose, followed by the encapsulation

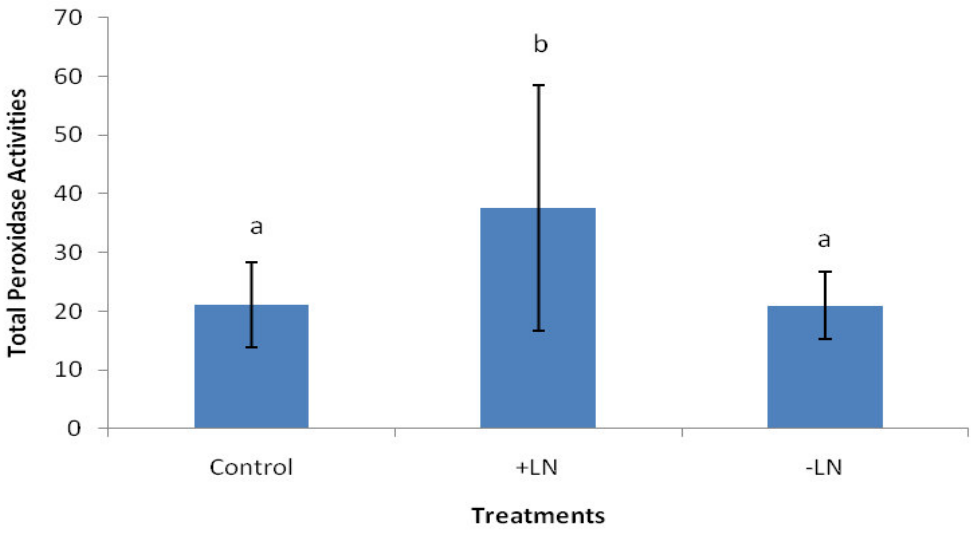

Figure 5. Effect of different treatments applied in encapsulation dehydration on total peroxidase activities of control, treated and cryopreserved PLBs. The error bars represent the standard deviation of means of 3 replicates.

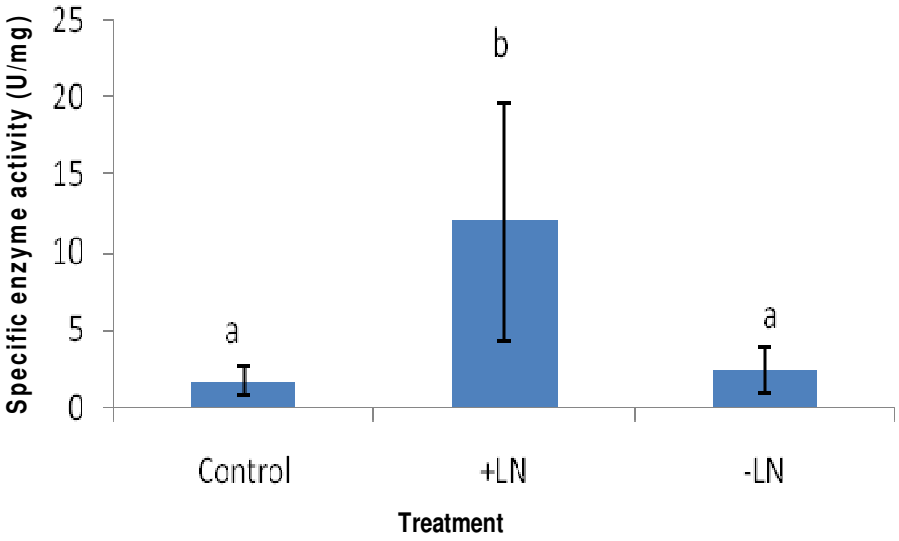

Figure 6. Effect of different treatments applied in encapsulation dehydration on specific peroxidase activities of control, treated and cryopreserved PLBs. The error bars represent the standard deviation of means of 3 replicates.

of the PLBs in 3.5% sodium alginate, prior to cryopreservation. Cryopreserved PLBs had produced the least chlorophyll and total soluble protein contents. However, the highest total and specific peroxidase activity was observed in cryopreserved PLBs.

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