African Journal of Biotechnology Vol. 10(18), pp. 3534-3539, 2 May, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.1128
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Early investigation on cryopreservation of *Dendrobium* sonia-28 using encapsulation-dehydration with modified Evan blue assay

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Accepted 14 March, 2011

This study was conducted to determine the potential of cryostoring and regenerating *Dendrobium* sonia-28 protocorm-like bodies (PLBs) using the encapsulation-dehydration technique. The parameters tested for this study included the PLB size range (1 to 2 and 3 to 4 mm), preculture using six different sucrose concentrations (0.0, 0.2, 0.4, 0.6, 0.8 and 1.0 M) and encapsulation using three different sodium alginate concentrations (2.5, 3.0 and 3.5%). Based on initial trials, 1 to 2 mm PLBs that were precultured in 1.0 M sucrose were selected for further studies as they produced the best viability as indicated by the Evans blue (EB) staining method. Subsequently, the PLBs were subjected to a 30 min encapsulation experiment involving the three sodium alginate concentrations. Finally, the chlorophyll content and total soluble protein of cryopreserved, non-cryopreserved and untreated PLBs were determined.

Key words: Orchid, protocorm-like bodies, cryopreservation.

INTRODUCTION

The Orchidaceae are one of the largest families among flowering plants having about 22,500 species under 779 genera (Mabberley, 2008). Many of the orchid species such as *Paphiopedilum, Cypripedium, Vanda*, etc. are said to be in extinction in their natural habitats as a result of horticultural collection. Many endangered orchid species have very small population sizes (less than 100 plants), and *in situ* conservation of orchids poses a big challenge to conservation management agencies (Johnston et al., 2009). The conventional way of conserving orchids is by collecting the living orchids and growing them in greenhouse or net house as well as by embryo culture (Pandey et al., 2008). Methods involving vegetative propagation are time consuming and high cost is required (Saiprasad and Polisetty, 2003). Hence, cryopreservation has been touted as a practical and promising tool for long-term orchid conservation (Johnston et al., 2009). The rapid destruction of natural habitats of orchid species as well as the heterozygosity and high cost of maintenance incurred from breeding stocks highlights the importance of developing reliable methods for germplasm storage (Ammirato et al., 1983). Problems may also arise when the selected plant germplasm cannot be stored as seeds because no seeds are formed, or seeds formed are recalcitrant in nature. In this light, unconventional preservation protocols such as slow growth storage and cryopreservation are the only practical methods to store the material (Keller et al., 2006). Cryopreservation is storage of explants at the temperature of liquid nitrogen (-196°C). The merits are the possibility of overcoming the problems of genetic instability and a reduction in the risk of contamination during subcultures (Goldner et al., 1991). To be successful in cryopreservation, the formation of ice crystal inside the cells must be avoided during immersion in liquid nitrogen (Shibli et al., 2001). The development of synthetic seed technology is now taken as an ideal alternative propagation method for several important agronomic and horticultural crops such as cotton, pines, seedless water-

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Abbreviations: PLB, Protocorm-like bodies; **EB,** Evans blue; **MS,** Murashige and Skoog media; **LN,** liquid nitrogen.

melon and banana. The synthetic seeds of orchids are formed by encapsulation of protocorm-like bodies (PLBs) in an alginate gel (Saiprasad and Polisetty, 2003).

The aim of this study is to establish a method of preserving the PLBs of *Dendrobium* sonia-28 through the encapsulation-dehydration technique.

The main objectives of this study are to determine the best PLB size range, to select the best sucrose concentration for the preculture of the PLBs, to determine the best sodium alginate concentration for encapsulation and to produce the highest viability of the PLBs in the cryopreservation experiment. These were followed by biochemical tests (chlorophyll determination and total soluble protein assay) as the post-cryopreservation analyses.

MATERIALS AND METHODS

Plant material

The *in vitro Dendrobium* sonia-28 PLB culture was propagated in the School of Biological Sciences, Universiti Sains Malaysia. The orchid cultures were subjected to the following room conditions: 25 ± 2°C, with 16 h photoperiod under cool white fluorescent lamps (Philips TLD, 36W) at 150 $\mu molm^{-2} s^{-1}$.

Media preparation

All media used in the experiment were prepared using half-strength MS (Murashige and Skoog, 1962) components. The pH values of the media were adjusted in the range of 5.7 to 5.8 prior to autoclaving.

Preculture of different sized PLBs in various concentrations of sucrose

The PLBs were excised into two different size ranges (1 to 2 and 3 to 4 mm) and precultured in half-strength MS semi-solid medium enriched with different concentrations of sucrose: 0 (control), 0.2, 0.4, 0.6, 0.8 and 1.0 M. The explants were stored at 25 ± 2°C under cool white fluorescent lamps (Philips TLD, 36W) for 24 h.

Encapsulation of precultured PLBs using different concentrations of sodium alginate

The precultured PLBs were dropped into sodium alginate prepared at the following concentrations: 2.5, 3.0 and 3.5% (w/v). Each PLB was aspirated with 100 µl of the alginate solution into a pipette tip modified to a diameter of 5 mm, and dropped into 0.1 M $CaCl_2$ solution supplemented with half-strength MS medium components. The beads were left to harden for 30 min, with occasional agitation of the culture vessel.

Osmoprotection of the encapsulated PLBs

After hardening, the beads were aseptically removed and placed in 100 ml conical flasks containing 25 ml liquid medium supplemented with 0.75 M sucrose. The explants were then placed on an orbital shaker set at 120 rpm for 24 h.

Dehydration of the encapsulated PLBs

The osmoprotected beads were strained out of the culture vessels, dabbed dry on sterile filter paper and then dehydrated for three hours in hermetically-sealed culture jars containing 50 g silica gel. The silica gel was previously heat-sterilised and reactivated overnight at 103°C using an oven.

Cryostorage of the encapsulated PLBs

The dehydrated beads were placed in 2 ml cryovials, which were then secured on cryocanes before direct storage in LN for 24 h. Thawing was performed at 40 ± 2°C for 90 s in a water bath, with rigorous shaking of the cryovials, followed by the placement of the beads onto the semi-solid recovery medium. Control experiments involved the direct transfer of dehydrated beads to recovery medium consisting of half-strength MS components, circumventing both the cryostorage and thawing steps. The recovering PLBs were kept in a dark room for four days in the culture room, followed by placement in dim light conditions, with the cultures covered using a white cloth. This is followed by direct exposure to direct light: 25 ± 2°C with 16 h photoperiod under cool white fluorescent lamps (Philips TLD, 36W) at 150 $\mu molm^{-2} s^{-1}$.

Evans blue (EB) dye assay

The viability of the PLBs was determined using Evans blue staining test. The absorption value of the supernatant was measured at 600 nm using a spectrophotometer (U-1900 HITACHI). Lower absorbance values indicate higher viabilities of the explants.

Chlorophyll content determination analysis

The chlorophyll content within the cryopreserved, non-cryopreserved and untreated PLBs was determined based on the Harbone method (Harbone, 1973).

Total soluble protein

The Bradford method (1976) was used to determine the protein content of cryopreserved, non-cryopreserved and untreated PLBs.

Statistical analysis

Each parameter tested consisted of three replicates, with each replicate containing 10 PLBs. Means obtained were subjected to one-way analysis of variance (ANOVA) and differentiated with Tukey's test.

RESULTS AND DISCUSSION

Preculturing of different-sized PLBs in various concentrations of sucrose

Preculturing in a sucrose-enriched medium is a necessary step necessary to increase the tolerance of the PLBs to LN. Preculture of plant material in media that contain sucrose or sorbitol increased the survival rate of shoot tips after cryostorage, in the presence of cryoprotectants (Yamada et al., 1991; Niino et al., 1992). Sucrose

preculture improves survival rate of post-thaw explants (Panis et al., 1996), and participates in the improvement of dehydration and freezing tolerance in plant tissues (Grapin et al., 2003).

Preculturing in media containing high sucrose concentrations was important for the survival of *Dendrobium* sonia-28 PLBs after cryopreservation, as indicated by the results obtained from the experiment. Figure 1 shows the absorbance values of cryopreserved PLBs at 600 nm. For the 3 to 4 mm PLBs, preculture in 0.4 M sucrose produced the lowest absorbance value (0.023). The 1 to 2 mm PLBs which were precultured in 1.0 M sucrose had the lowest absorbance value (0.019). Hence, the smaller-sized PLBs were selected for the next experiment since the viability rate was the highest (lowest reading) by using Evan blue staining test. The advantages of sucrose as preculture treatment in cryopreservation could be attributed to two effects (Steponkus et al., 1992). First, sucrose, like other osmotic active substances, has an osmotic dehydration effect, as it reduces water content in tissues (Tanaka et al., 2004). Second, sucrose is able to enter cells (Dumet et al., 1993). The theory that sucrose is able to penetrate cells has been proven by histological observations of intracellular starch accumulation during preculture (Gonzalez-Arnao et al., 1993). The accumulation of sucrose within tissues enhances viability when freezable water is removed to the point of reaching glassy states during vitrification in the presence of LN (Steponkus et al., 1992). When high concentration of sucrose was added to the preculture medium, it was predicted to both promote intracellular dehydration through the osmotic effect and maintain the integrity of the plasma and the inner membrane during the freezing process (Blakesley et al., 1996).

Encapsulation of precultured PLBs using different concentrations of sodium alginate

The encapsulation concentration experiment indicated that 3.0% sodium alginate was the best in the encapsulation of the PLBs (Figure 2) as the absorbance value obtained at this point was the lowest (0.025). Saiprasad (2001) stated that optimum capsule hardness and rigidity is obtained from the complexation of 3.0% sodium alginate with 75 mM $CaCl_2.2H_2O$ for 30 min, as evidenced in this experime Sodium alginate concentrations play a crucial role in obtaining beads with optimum hardness and rigidity. The alginate capsule also protects the explant within from mechanical injury (Saiprasad, 2001). At 3% (w/v), the solution has low viscosity, at 250 cps (Gonzalez-Arnao and Engelmann, 2006; Reed, 2008). Higher concentrations (5, 6 and 7%) were too viscous and the beads formed were harder and hindered the emergence of shoot and root. The possible reasons for this inhibition are the unsuitable elasticity of the gel bead and oxygen deficiency within the gel bead (Saiprasad and Polisetty, 2003).

Chlorophyll content determination

Chlorophyll is a pigment which is found in plants, and gives the green colour; it absorbs the light crucial for photosynthesis. There are two main types of chlorophyll, *a* and *b,* both of which differ by their side chain composition. Chlorophyll *a* is the main photosynthetic pigment of aerobic organism. All photosynthesizing plants, algae and cyanobacteria contain chlorophyll *a*. Chlorophyll *b*, found only in green algae and in plants, functions mainly to absorb light and transfer it to chlorophyll *a* (Devesa-Rey et al., 2008).

Figure 3 indicates that the total chlorophyll *a* for the PLBs of *Dendrobium* sonia-28 in all treatments were lower than chlorophyll *b*. The control PLBs gave the highest chlorophyll content, in both chlorophyll *a* and *b* (Figure 3). Cryopreserved PLBs gave the lowest chlorophyll content for both chlorophyll *a* and *b*. Observations of photosynthetic activity in cryopreserved PLBs indicates that cryostorage inhibits activities of both photo systems to certain level (Safrinah et al., 2009). This might be a factor for the lack of regeneration in cryopreserved materials after its transfer to normal tissue culture conditions. Despite strong inhibition of photosynthetic electron transport, observations indicate that the deep-freezing does not contribute to the immediate death of the PLBs (Safrinah et al., 2009).

Total soluble protein analysis

The Bradford method (1976) was used to determine the protein content of the PLBs. The Bradford method was chosen because it is simple, fast and low in cost when compared to other methods (Nuria and Pilar, 2001). When Coomassie Brilliant Blue G-250 (an acidic solution of dye) is added to a solution of protein, the colour of the solution changes from a reddish brown to blue as the dye binds to the protein via electrostatic attraction between the dye's sulfonic groups to the protein. Apart from that, when the acidic dye solution binds to protein, its peak absorbance value changes from 465 to 595 nm. Therefore, the measurement of the absorbance of the protein-dye complex at 595 nm allows an accurate quantification of the protein content of a sample (Nuria and Pilar, 2001).

Cryopreserved PLBs had the lowest total soluble protein content when compared to untreated PLBs which had the highest value of total soluble protein content (Figure 4). Nuria and Pilar (2001) reported that this condition occurs since a plant will experience a reduction in terms of growth and metabolic activities when subjected to any biotic or abiotic stress factor. Moreover, there is a high possibility that the PLBs intended for cryopreservation possess

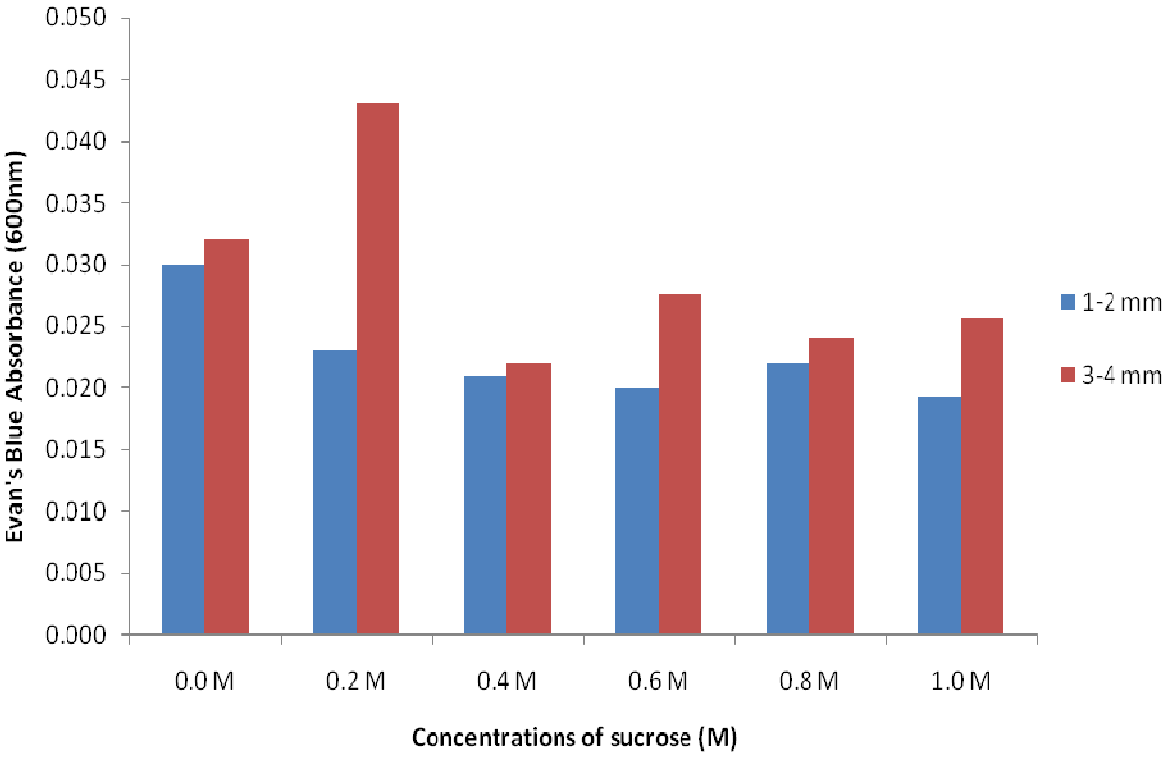

Figure 1. Treatment of PLBs *Dendrobium* sonia-28 with six sucrose concentrations. Two sizes of PLBs (1 to 2 and 2 to 3 mm) were precultured in six sucrose concentrations for 24 h. The viability of the PLBs was tested using the Evans blue staining method

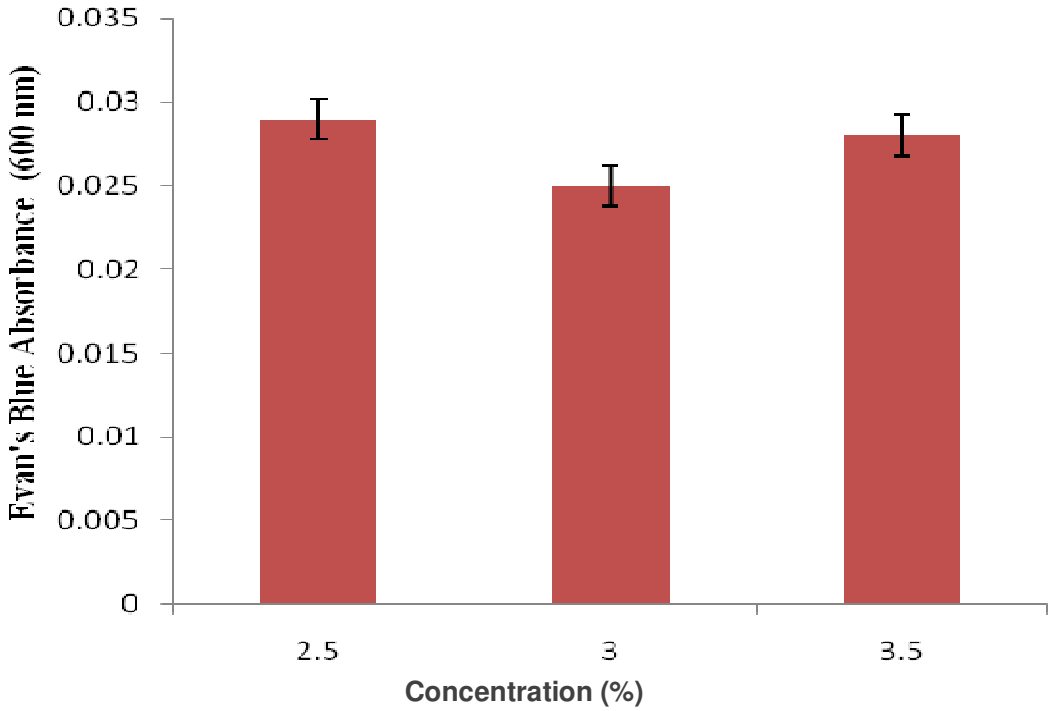

Figure 2. Encapsulation of PLBs with different concentrations of sodium alginate. Three sodium alginate concentrations (2.5, 3.0 and 3.5%) were used and the beads were left to harden for half an hour in 0.1 M $CaCl_2$.

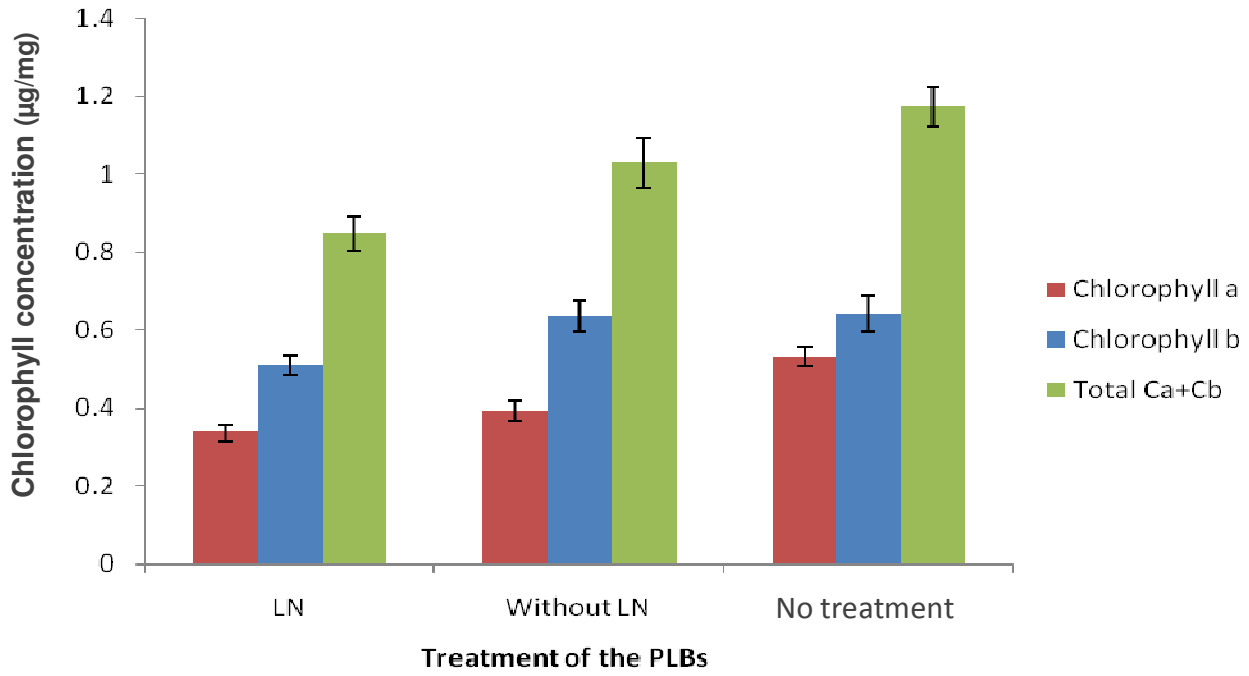

Figure 3. Concentrations of chlorophyll *a* and *b* and total chlorophyll *a* and *b* in PLBs of *Dendrobium* sonia-28. Chlorophyll determination was carried out in order to analyse the chlorophyll content in cryopreserved PLBs, non-cryopreserved PLBs and untreated PLBs of *Dendrobium* sonia-28.

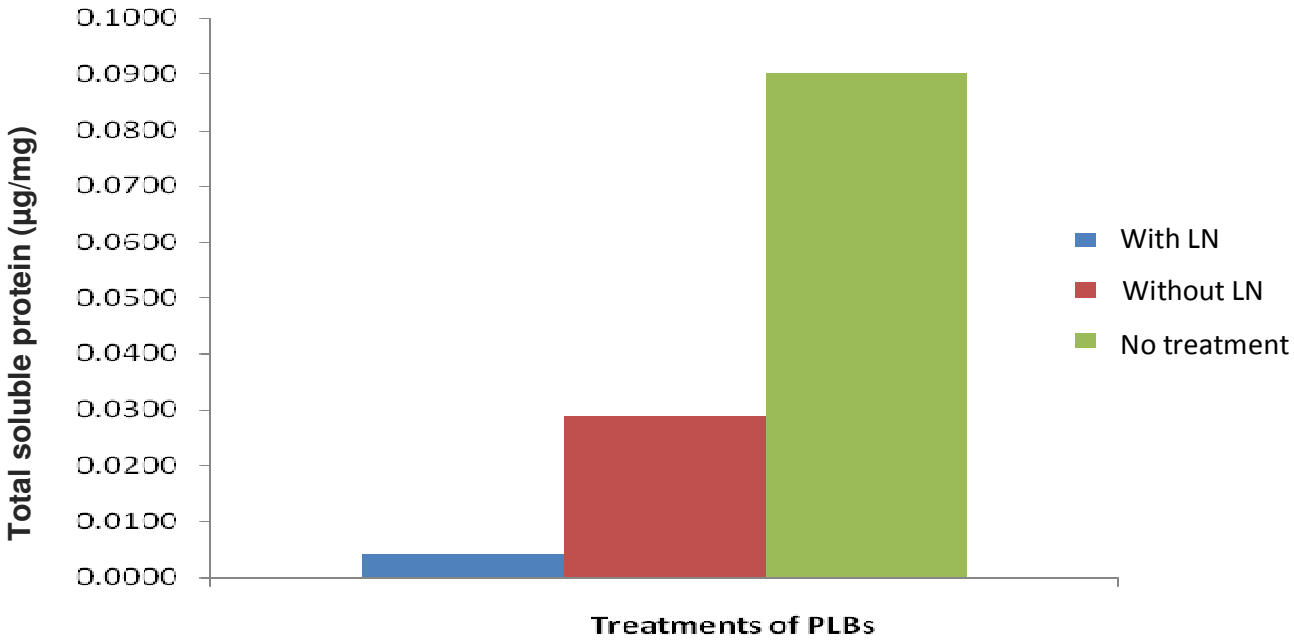

Figure 4. Total soluble protein content in PLBs of *Dendrobium* sonia-28 under three different treatments. After two weeks of recovery, cryostored, non-cryopreserved and untreated 1 to 2 mm PLBs were tested for total soluble protein.

inadequate or no stress tolerance mechanisms. In conclusion, the low protein concentrations should be interpreted as a clear sign of stress damage in plant cells (Nuria and Pilar, 2001).

Conclusion

From this study, it was observed that 1 to 2 mm PLB size produced the best viability after a 24 h preculture in semi-

solid half-strength MS media containing 1.0 M sucrose. The best result for PLB encapsulation was obtained when 3.0% (w/v) sodium alginate was used with 0.1 M $CaCl_2$, followed by a 30 min complexation period. Untreated PLBs of *Dendrobium* sonia-28 produced both the highest concentration of both chlorophyll *a* and *b* in the chlorophyll test and the highest total soluble protein content in the total soluble protein analysis.

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