http://biology.num.edu.mn
http://mjbs.100zero.org/
Volume 13(1-2), 2015

Mongolian Journal of Biological Sciences

ISSN 1684-3908 (print edition)
MJBS
ISSN 2225-4994 (online edition)

Original Article

Cryopreservation of PLBs of *Brassidium* Fly Away Using Encapsulation-Dehydration Technique

Arulvilee Rajasegar[1,2], Ranjetta Poobathy[1], Xavier Rathinam[2], Yungeree Oyunbileg[3] and Sreeramanan Subramaniam[1]*

[1]School of Biological Sciences, Universiti Sains Malaysia (USM), Georgetown, 11800, Penang, Malaysia
[2]AIMST University, Semeling, 11800, Kedah, Malaysia.
[3]Institute of General and Experimental Biology, Mongolian Academy of Sciences, Peace avenue-54B, Ulaanbaatar 13330, Mongolia.

Abstract

Key words: Encapsulation-dehydration, PLBs, cryopreservation

Article information:
Received: 29 Dec. 2013
Accepted: 27 Mar. 2015
Published: 27 Nov. 2015

Correspondence*: sreeramanan@gmail.com; sreeramanan@usm.my

In vitro grown protocorm-like bodies (PLBs) of *Brassidium* Fly Away orchid hybrid were cryopreserved using encapsulation- dehydration technique. The viability of the cryopreserved cells was determined by 2,3,5-triphenyltetrazolium chloride (TTC) assay. For the preculture treatment, the PLBs were excised into two standard sizes of 1-2 and 4-5 mm and were precultured on half-strength Murashige and Skoog (MS) semi solid medium supplemented with different concentrations of sucrose (0, 0.2, 0.4, 0.6, 0.8 and 1.0M). The PLBs size 4-5 mm and 0.6 M sucrose concentration was selected based on highest viability obtained in TTC assay. The PLBs were encapsulated for 30 minutes using 3% (w/v) liquid sodium alginate medium supplemented with 0.4M sucrose and 0.1M calcium chloride and osmoprotected in 0.75M sucrose solution for 24 hours at 25°C. The beads were then dehydrated using 50g heat-sterilised silica gel for four hours, cryopreserved for 24 hours, thawed in a 40±2°C water bath for 90 seconds, and regenerated in semi-solid half-strength. Biochemical analyses were conducted and the cryopreserved PLBs had produced lower content of chlorophyll while the highest specific peroxidase activity was observed in cryopreserved PLBs.

Cite this paper as: Rajasegar, A., Poobathy, R., Rathinam, X., Oyunbileg, Yu. & Subramaniam, S. 2015. Cryopreservation of PLBs of *Brassidium* Fly Away using encapsulation-dehydration technique. *Mong. J. Biol. Sci*., 13(1-2): 19-23.

Introduction

The Orchidaceae is one of the largest families of flowering plants and become popular in floricultural industry because of their colours, shapes, sizes, and bloom persistence (Yu & Xu, 2007). The growing demands for orchid cut flowers act as a boost to the various breeding programmes of orchids. Orchids are threated because of the unstoppable harvesting of wild type orchids, which harm the existence of the wild species of orchids. The genetic resources of ornamental plants, especially orchids are required to be stored due to their increasing of extinction.

Cryopreservation is an alternative or a duplicate storage for the traditional *in situ* and *ex situ* germplasm conservation (Engelmann *et al*., 2000). The successful cryopreservation of biological tissues can be achieved by avoiding the intracellular ice crystal formation due to an irreversible damage to cell membranes will occured and thus destroying their semi-permeability (Panis *et al*., 2005). Cryogenic technique such as vitrification, encapsulation-dehydration and encapsulation-vitrification has been developed and the number of species or

cultivator that has been cryopreserved has widely increased (Halmagyi *et al*., 2005). Encapsulation-dehydration is one of the efficient techniques and is based on the technology developed for producing synthetic seed, such as encapsulation of explants in calcium alginate beads (Reed, 2008). This technique has been applicable to a wide range of plant material, such as rasberry (Wang *et al*., 2005), Yam (Hirai & Sakai, 2011) and *Artemisia* herba-alba (Sharaf *et al*., 2012).

Brassidium Fly Away is one of the hybrids in *Brassia* genus from the hybridization between *Brassidium* Gilded Urchin and *Oncidium maculatum.* Single protocorm-like bodies (PLBs) of *Brassidium* Fly Away are suitable target material for cryopreservation experiments. PLBs are attractive explant to be used as target tissue for cryopreservation for their easiness to be propagated *in vitro* which provide plenty of material to work with and they proved to be a reliable material of potentially regenerable tissue (Ishikawa *et al*., 1997). Therefore, this study was carry to establish cryopreservation of *Brassidum* Fly Away using PLBs encapsulation-dehydration technique.

Material and Methods

Plant material. Protocorm-like bodies of *Brassidium* Fly Away induced from *in vitro* protocorm cultures were obtained from a commercial orchid nursery. The hybrid of orchid was used as the starting plant material to initiate multiplication of PLBs for this study. The *Brassidium* Fly Away was subjected to temperature of 25±2°C with 16h photoperiod under cool white fluorescent lamps (Philips TLD, 36W) at 150 µmol $m^{-2}s^{-1}$ in the tissue culture room.

Encapsulation-dehydration technique. *Effect of sucrose concentration with different PLB size.* The PLBs were excised into two different sizes 1-2 and 4-5 mm and precultured in half-strength MS semi-solid medium enrich with different concentration of sucrose (0, 0.2, 0.4 , 0.6, 0.8 and 1.0 M). Ten (10) PLBs were placed in the Petri dishes containing the preculture media and then sealed with parafilm. The Petri dishes were stored in culture room at 25±2°C under cool white fluorescent lamps for 24 hours preculture time.

Effect of alginate concentrations. The PLBs were taken out aseptically and inserted into universal bottle containing sodium alginate 3.0% by using forceps. Micropipette of 100-1000 µL was adjusted to 100 µL and cut tip was fixed on it. The PLBs were placed into the $CaCl_2 \cdot 2H_2O$ solution and the forming beads were immersed for 30 minutes. These steps were repeated using different concentrations of alginate solution 2.5 and 3.5%.

Effect of osmoprotection concentrations. After 30 minutes immersion in the $CaCl_2 \cdot 2H_2O$ solution, the beads were taken out aseptically and put into conical flask which contains osmoprotection media by using forceps. The conical flask was flamed and sealed with parafilm and placed on a shaker (120 rpm) overnight in the tissue culture room. This process repeated for osmoprotection concentration 0.5, 0.75 and 1.0 M. Culture jars containing 50 g silica gel was flamed and a filter paper was placed in it. The beads were put in the silica gel and the culture jars was sealed with parafilm. The beads were let in the silica gel for 3 hour.

Cryostorage of the beads in liquid nitrogen and thawing. After dehydration of silica gel in 3 hours period, the beads were transferred into plastic cryovial (1.8 mL). Then the plastic cryovial were plunged into LN tank for one day. PLBs that were subjected to without LN treatment were directly transferred from the silica gel into growth recovery (GR) media. After 24 hours, the cryovials containing cryopreserved PLBs were taken from the LN tank and immediately thawed in autoclave distilled water at 40±2°C for 90 s with slight shaking. The beads were placed on half-strength MS semi-solid medium, and left to incubate in the dark for five days, the second week under dimmed lighting (3.4 µmol.m^{-2}.s^{-1}) and the PLBs were incubated at 25±2ºC under 16

hours photoperiod using cool white fluorescent lamps (Philips TLD, 36W, 150µmol.m-^{2}.s^{-1}) in the third week.

2,3,5-Triphenyltetrazolium chloride (TTC) Assay. The viability of the PLBs was determined by using TTC staining. The method is based on the reduction of colourless TTC into red formazan. The PLBs are immersed in TTC solution for 15 to 20 hours. The TTC solution was drained off and the cells were washed with distilled water. After that, cells were vortexes and extracted with 7 ml of 95% ethanol in water bath at 80°C for 5 min. The extracted was cool and made up to 10 ml with 95% ethanol. The absorbance was measured by using spectrophotometer at 490 nm.

Chlorophyll determination analysis. The slight modification of method by Harbone (1973) was used to determine the chlorophyll content in the cryopreserved, non-cryopreserved PLBs and stock culture.

Peroxidase activity. The peroxidase assay was modified from Flocco and Giulietti (2003).

Statistical analysis and experimental design. The experiments were conducted in a randomized design and the samples consisted of three replicates containing 10 explants for each parameter tested. Means in the other experiments were analyzed through the one-way analysis of variance (ANOVA) and differentiated with Tukey's test, with the confidence intervals at 95%.

Results

In this study, the following analysis was performed using PLBs of *Brassidium* Fly Away that were precultured in semi-solid MS medium with different sucrose concentration (0, 0.2, 0.4, 0.6, 0.8 ,1.0 M) and PLBs of different sizes 2-3 mm and 4-5 mm for 24 hours. Figure 1 shows the PLB with larger size 4-5 mm that was treated in 0.6 M of sucrose had recorded the highest absorbance value. The viability of observation of PLBs is evaluated based on the estimation of the amount of formazan produced from the reduction of TTC due to action of dehydrogenases in the living cells tissues. PLB with size 4-5 mm were chosen for this experiment due to the ability withstand the entire encapsulation technique better than smaller PLBs (Fig. 2).

Concentration of sodium alginate plays a crucial role in obtain beads with optimum hardness and rigidity. This proof is tallied with the result of this experiment which shows 3.0% of the alginate solution gave the highest viability compared 2.5 and 3.5% of alginate concentration (Fig. 3). Osmoprotection for encapsulation dehydration starts with increasing sucrose concentrations. Controlled rate cooling protocol provide some osmoprotection by slow addition of cryoprotectant solution. Osmoprotection concentration of 0.75 M produced the highest record of viability (Fig. 4).

Discussion

Pretreatment, also known as preculture involves parameters, such as the type of chemicals used in the treatment (sugar or polyols), the

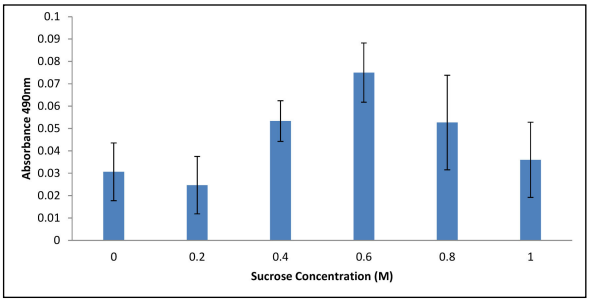

Figure 1. Effects of different sucrose concentration on viability of *Brassidium* Fly Away PLBs. The error bars represent the standard deviation of means of 3 replicates.

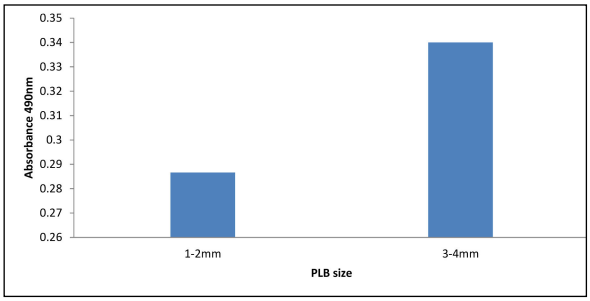

Figure 2. Effects of PLB size on surivival of *Brassidium* Fly Away.

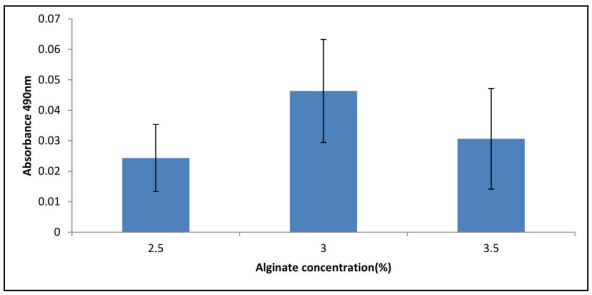

Figure 3. Effects of different alginate concentration on viability of *Brassidium* Fly Away PLBs. The error bars represent the standard deviation of means of 3 replicates.

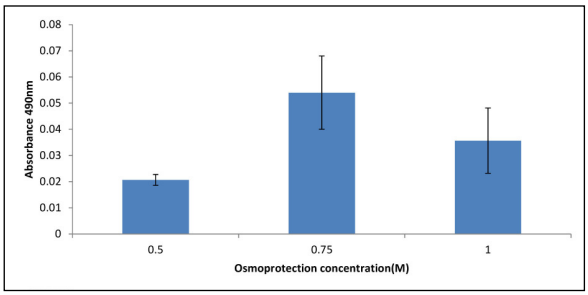

Figure 4. Effects of different osmoprotection concentration on viability of *Brassidium* Fly Away PLBs. The error bars represent the standard deviation of means of 3 replicates.

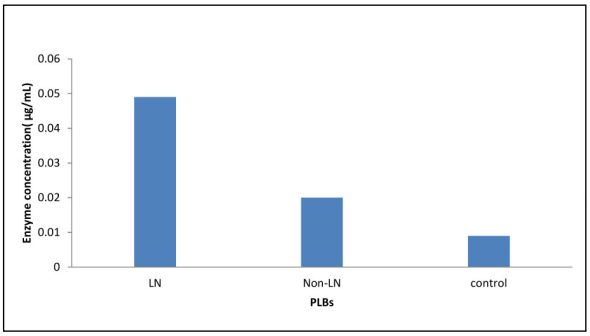

Figure 5. Effects of peroxidase enzyme of control, treated and cryopreserved PLBs of *Brassidum* Fly Away in encapsulation-dehydration.

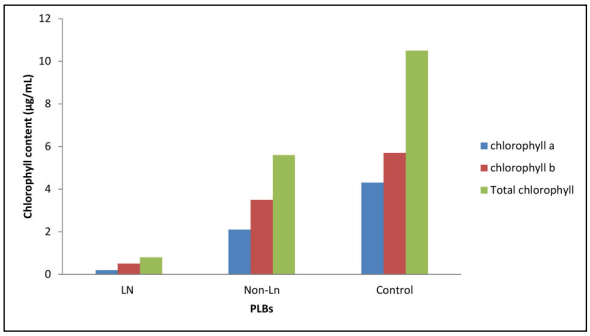

Figure 6. Effects of variuos tretaments on the chlorophyll contents of cryopreserved, treated and untreated PLBs in encapsulation-dehydration experiment.

duration of the pretreatment, and the method of conducting the pretreatment. In order to increase the tolerance to liquid nitrogen, the pretreatment step supplemented with sucrose is essential. Sucrose participates in the improvement of dehydration and freezing tolerance in plant tissues (Grapin *et al*., 2003).

In general, 3.0% sodium alginate upon complexation with 75 mM $CaCl_2 \cdot 2H_2O$ for 30 minutes comes out with optimum capsule hardness and rigidity (Saiprasad, 2001). It was also recommended that suitable combinations of nutrients, growth regulators and protectants were added to alginate beads to produce better growth yields in explants (Verma *et al*., 2010).

Water availability is crucial for the development of all living cells. Various physical and chemical parameters, such as desiccation and hyperosmotic stress generate cellular dehydration. The mechanisms of cellular adaptation preventing water loss under hyperosmotic conditions (osmoregulation) have been extensively studied in plant.

Chlorophyll determination. Chlorophyll is the pigment which found in plants that give the green colour and absorbs the light crucial for photosynthesis. There are two main types of chlorophyll, named *a* and *b*. The composition of side chain (in chlorophyll *a* it is $–CH_3$, in *b* it is CHO), makes them slightly differ. These both chlorophylls are very effective photoreceptors because they contain a network of alternating single and double bonds and the orbitals can delocalize to stabilize the structure. Chlorophyll *a* is the main photosynthetic pigment of aerobic organism. Based on graph that was obtained shows the control gave the highest chlorophyll content, in both chlorophyll *a* and *b*. Cryopreserved PLBs gave the lowest chlorophyll content while non-cryopreserved shows moderate the chlorophyll content (Fig. 6). The cryopreserved PLBs formulate that the deep-freezing technique develops thus far inhibits activities of botch photo systems to certain level (Safrinah *et al*., 2009). This might be a potential factor for the lack of regeneration of plant material after its transfer to normal conditions.

Peroxidase activity. Peroxidase is a type of homoprotein that are ubiquitous and function in catalyzing the oxidation of a wide variety of substrates by using hydrogen peroxide (H_2O_2) (Flocco & Giulietti, 2003). The highest peroxidase activity was from the treatment of PLBs with liquid nitrogen and followed by non-cryopreserved and the lowest observed peroxidase activity was for PLBs in control condition (without any treatment) (Fig. 5). 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 (peroxidases) released under these conditions.

Conclusion

The best conditions for the encapsulation-dehydration of *Brassidium* fly away is the preculture of 4 to 5 mm PLBs in half-strength semi-solid MS medium supplemented with 0.6 M sucrose, followed by the encapsulation of the PLBs in 3.0% sodium alginate with 0.75 M osmoprotection media, prior to cryopreservation. Cryopreserved PLBs had produced lower content of chlorophyll and most damages resulting from cryostorage occurred in the cell wall and nucleus of the cells while the highest specific peroxidase activity was observed in cryopreserved PLBs.

Acknowledgements

The authors would like to thank Universiti Sains Malaysia Research Grant (USM-RU 2012) for supporting this project.

References

Engelmann, F. 2000. Importance of cryopreservation for the conservation of plant genetic resources. In Engelmann, F. and Takagi, H. (Eds.): *Cryopreservation of Tropical Germplasm: Current Research Progress and Application.* pp. 8–20, JIRCAS, Rome.

Flocco, C. G. & Giulietti, A. M. 2003. Effect of chitosan on peroxidase activity and isoenzyme profile in hairy root cultures of *Armoracia lapathifolia*. *Applied Biochemistry and Biotechnology,* 110: 175-183.

Grapin, A., Dumet, D., Holota, H., & Durion, N. 2003. Cryopreservation of *Pelargonium* shoot tips by encapsulation-dehydration. Effect sucrose concentration, dehydration duration and genotype. *Acta Horticulturae*, 623: 225-230.

Halmagyi, A., Deliu, C. & Coste, A. 2005. Plant regrowth from potato shoot tips cryopreserved by a combined vitrification-droplet method. *CryoLetters*, 25: 313-322

Harbone, J. B. 1973. Photochemical Methods. Chapmann and Hall Ltd., London, pp. 49-188.

Hirai, D. & Sakai, A. 2001. Recovery growth of plants cryopreserved by encapsulation–vitrification. *Bull. Hokkaido Prefect. Agric. Exp. Station*, 80: 55–64

Ishikawa, K., Harata, K., Mii, M.; & Sakai, A., Yoshimatsu, K. & Shimomura, K. 1997. Cryopreservation of zygotic embryos of a Japanese terrestrial orchid (*Bletilla striata*) by vitrification. *Plant Cell Reports*, 16: 754–757.

Murashige, T. & Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiologiae Plantarum*, 15: 473-497.

Panis, B., Piette, B. & Swennen, R. 2005. Droplet vitrification of apical meristems: a cryopreservation protocol applicable to all Musaceae. *Plant Science*, 168(1): 45-55.

Reed, B. M. 2008. Cryopreservation—Practical Considerations. In: Reed, B. M. (Ed.), *Plant Cryopreservation: A Practical Guide*. pp. 3-13, Springer.

Safrinah, R., Xavier, R., Uma Rani, S. & Sreeramanan, S. 2009. Optimisation of cryopreservation technique in *Mokara* golden nugget orchid using PVS2 vitrification. *International Journal of Agricultural Research*, 4: 218-227.

Saiprasad, G. V. S. 2001. Artificial Seeds and Their Applications. Resonance, May 2001.

Sharaf, S. A., Shibli, R. A., Kasrawi, M. A. & Baghdadi, S. H. 2012. Cryopreservation of wild Shih (*Artemisia* herba-alba Asso.) shoot-tips by encapsulation-dehydration and encapsulation–vitrification. *Plant Cell, Tissue and Organ Culture*, 108: 437–444.

Verma, S. K., Rai, M. K., Asthana, P., Jaiswal, V. S. & Jaiswal, U. 2010. *In vitro* plantlets from alginate-encapsulated shoot tips of *Solanum nigrum* L. *Scientia Horticulturae*, 124: 517-521.

Wang, Q., Laamanen, J., Uosukainen, M. & Valkonen, J. P. T. 2005. Cryopreservation of in vitro-grown shoot tips of raspberry (*Rubus idaeus* L.) by encapsulation–vitrification and encapsulation–dehydration. *Plant Cell Reports*, 24: 280–288.

Yu, H. & Xu, Y. 2007. Biotechnology in Agriculture and Forestry, Vol. 61. *In:* Pua, E.C and Davey, M.R (eds). *Transgenic Crops* VI. Springer Berlin. Pp. 273-288.

*****