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Full Length Research Paper

Fundamental concept of cryopreservation using *Dendrobium* sonia-17 protocorm-like bodies by encapsulation-dehydration technique

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This study was carried out to evaluate the potential of using the encapsulation-dehydration technique on cryopreservation protocorm-like bodies (PLBs) of *Dendrobium* sonia-17. The survival of the PLBs was assessed based on the effects of 4 dehydration periods (0, 1, 3 and 5 h) and 4 different concentrations of 24-h sucrose pretreatment (0, 0.3, 0.5 and 0.7 M). Upon dehydration, moisture content was determined and the PLBs were evaluated for survival using absorbance values from 2, 3,5-triphenyltetrazolium chloride (TTC) assay at 530 nm and regeneration observations. Moisture content declined with the dehydration time, but the decline was not significant for encapsulated PLBs. All cryopreserved PLBs gave very low survival irrespective of the dehydration period. The best survival percentage in the cryopreservation of the PLBs of *Dendrobium* sonia-17 was obtained when the combination of 0.5 M sucrose pretreatment and 3 h dehydration time was applied in the experiment.

Key words: *Dendrobium* sonia-17, protocorm-like bodies, encapsulation-dehydration, 2,3,5-triphenyltetrazolium chloride (TTC) assay.

INTRODUCTION

Orchids are perceived as one of the most beautiful group of flowers among all flowering plants. Orchid diversity is incredible as they make up the largest family of flowering plants on earth, with about 800 genera, 20,000 different species and at least 200,000 hybrids (Lurswijidjarus and Thammasiri, 2004). Orchids are commonly propagated using the tissue culture technique from either the seed, which forms protocorm or from organogenesis leading to the formation of protocorm-like-bodies. The protocorm-like-bodies can be stored for long term using cryopreservation in order to preserve plant materials.

Cryopreservation, involves the storage of explants at ultra-low temperature of liquid nitrogen (-196°C). Metabolic and cellular processes are effectively stopped at such low temperature and thus allow the tissue to be in a state of suspended animation (Dereuddre et al., 1988). Cryopreservation has been applied on various orchid plant parts such as somatic embryos, shoot tips, cell suspension, protocorms, apical meristems and nodal suspension (Thammasiri, 2000; Bian et al., 2002; Nabila et al., 2009; Safrinah et al;., 2009).

Encapsulation-dehydration is a technique developed based on the production of synthetic seeds (Redenbaugh et al., 1986; Engelmann et al., 2008). The technique protects explants from conditions that are usually damaging to non-encapsulated samples when they are exposed to extreme treatments such as pretreatment in high sucrose concentrations and dehydration to low water contents. Intracellular ice formation is prevented during rapid exposure to liquid nitrogen as vitrification of the internal solutes of the samples takes place due to the removal of freezable water during the pretreatment and dehydration procedures (Engelmann et al., 2008; Engelmann, 1997). This results in high survival rates and direct regrowth of the samples during regeneration, as the explants remain largely intact after the thawing step (Engelmann et al., 2008; Engelmann, 1997). The encapsulation-dehydration

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protocol has also been successfully applied to more than 70 plant species worldwide (Engelmann et al., 2008).

The success of a cryopreservation protocol is largely dependent on the physiological and developmental state of the target tissues. Generally, the material chosen should be young, meristematic and totipotent in origin since they are most likely to have higher degree of tolerance towards low temperature condition. These cells should be small, contain only a few vacuoles with small amounts of water, and exhibit dense cytoplasm with high nucleo-cytoplasmic balance. The excised tissues, such as the embryos, orchid protocorms and embryonic axes, should be at a suitable growth stage in order to acquire dehydration and freezing tolerance, with the ability to regenerate vigorous plantlets after cryogenic storage (Nabila et al., 2009; Normah and Makeen, 2008). Protocorm-like bodies (PLBs) are attractive as target tissues for cryopreservation because of two main criteria: they are easily propagated *in vitro*, providing plenty of materials to work with and they proved to be a reliable source of potential regenerable tissues.

The aim of this study was to evaluate the effects of dehydration and sucrose preculture on the survival of non-encapsulated and encapsulated PLBs of *Dendrobium* sonia-17, with and without cryopreservation.

MATERIALS AND METHODS

Plant material

The *in vitro*-grown PLBs of *Dendrobium* sonia-17 orchid was used in this study (Figure 1). Stock cultures were cultured in half strength Murashige and Skoog media (Murashige and Skoog, 1962) supplemented with 1 mg/L benzylaminopurine BAP, 2% sucrose and 2.75 g/L gelrite. The cultures were incubated at 25 ± 2°C in a 16 h photoperiod under cool white fluorescent lamps (Philips TLD, 36 W) at 150 µmol m^{-2} s^{-1}.

Encapsulation of PLBs of *Dendrobium* sonia-17

The encapsulation method was modified from the method proposed by Sakai in 1995 (Sakai, 1995). PLBs measuring 3 to 5 mm in length were carefully excised and transferred to semi-solid half-strength Murashige-Skoog (MS) medium (Murashige and Skoog, 1962) devoid of BAP for 24 h prior to the cryopreservation experiments. They were then immersed in calcium-free half-strength liquid MS medium containing 3% alginic acid. Each PLB was dropped into liquid 0.1 M calcium chloride medium with a micropipette fitted with a tip modified to a width of 4 mm. Beads (5 mm) were left to harden in the solution for 30 min, with occasional agitation (Orbital shaker, N-BIOTEK Inc). The beads were then washed with liquid MS medium and transferred to empty Petri dishes.

Effects of dehydration on the survival of non-encapsulated and encapsulated PLBs of *Dendrobium* sonia-17 with and without cryopreservation

The non-encapsulated PLBs and encapsulated PLBs were dehydrated aseptically for 0 (control), 1, 3 and 5 h with 50 g of oven sterilized silica gel. After each dehydration period, two replicates of five PLBs were used for actual moisture content determination. Five replicates acted as the control and another five replicates were placed in sterile cryovials secured to cryocanes, and directly plunged in liquid nitrogen (MVE LAB50) for at least 16 h (+LN) and thawed rapidly in a water bath set at 38 to 40°C. Survival was estimated using the quantitative 2,3,5-triphenyltetrazolium chloride (TTC) method (Steponkus and Lanphear, 1967), with data recorded as the absorbance at 530 nm. Each experiment consists of 6 replicates per treatment with 10 samples each.

Effect of sucrose pretreatment (0, 0.3, 0.5 or 0.7 M) and dehydration on the survival of the PLBs of *Dendrobium* sonia-17 with and without cryopreservation

PLBs were precultured on various concentrations of sucrose (0, 0.3, 0.5 or 0.7 M) for 24 h and then dehydrated aseptically for 0 (control), 1, 3 and 5 h. After each dehydration period, the PLBs were removed for actual moisture content determination and for survival determination using the TTC method both prior and after the exposure to liquid nitrogen. The cryovials were immersed for 16 h in liquid nitrogen before thawing was carried out. Survival was immediately estimated using the quantitative TTC method, with data recorded being the absorbance at 530 nm. Each experiment consists of 6 replicates per treatment with 10 samples each.

Determination of moisture content

Two replicates of five PLBs were placed in an aluminium boat. The weight of the boat was first recorded, followed by the measurement of the weight of the boat with PLBs, using a 4-decimal point electrical balance (Pioneer^{TM}, OHAUS). The boat containing the PLBs was placed in an oven at 130°C for 1 h. Upon removal from the oven, the PLBs were placed in a desiccator to cool. The boat containing the dried PLBs was reweighed after 20 min. Moisture content on weight basis was determined as the loss of weight from the boat containing the PLBs and was expressed as the percentage (%) of the initial fresh weight.

The formula used to calculate the percentage moisture content was:

$$\text{Percentage moisture content (MC)} = \frac{(W_2-W_3)}{(W_2-W_1)} \times 100$$

Where, W_1 is the weight of aluminium boat (g); W_2 is the weight of aluminium boat + encapsulated PLBs before drying and W_3 is the weight of aluminium boat + encapsulated PLBs after drying.

All PLBs were carefully excised out of the capsule in order to determine the moisture content as compared to the non-encapsulated PLBs which cannot be made otherwise.

Regeneration

The beads were then cultured on half strength semi-solid MS media supplemented with 2% sucrose without the presence of any growth regulators for 3 weeks before analyzing using the TTC Assay. The beads were kept in dark condition at the 1st week; under a dim light (95 lux) condition at the 2nd week and were exposed to 16 h photoperiod (cool white fluorescent lamps (Philips TLD, 36 W) at 150µmol.m^{-2}.s^{-1}]) at the 3rd week.

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

In a modified protocol (Steponkus and Lanphear, 1967), PLBs

Figure 1. Stock culture of *in-vitro* grown PLBs of *Dendrobium* sonia-17 (Bar = 1.0 cm).

Table 1. Moisture content (%) of non-encapsulated and encapsulated PLBs of *Dendrobium* sonia-17 after each dehydration period prior to exposure to LN

Dehydration period (hour)	Moisture content (%)	
	Non-encapsulated PLBs	Encapsulated PLBs
0	84.0[a]	87.0[a]
1	77.7[b]	86.4[a]
3	77.0[b]	83.1[a]
5	58.7[c]	82.5[a]

Means with same letter(s) are not significantly different at $p < 0.05$ based on DMRT.

weighing 150 mg (fresh weight) were placed in 3 ml of TTC solution (0.18M TTC in 0.05 M $Na_2HPO_4.2H_2O$ and 0.05 M KH_2PO_4 buffer, pH 5.7) and incubated for 15 h at 30°C. The TTC solution was then drained and the PLBs were rinsed with distilled water. The PLBs were centrifuged in 7 ml of 95% ethanol at 2900 rpm for 10 min. The extract was placed in a water bath at 80°C for 5 min, followed by the addition of 3 ml of 95% ethanol after the extract was cooled. The absorbance values of the extracts were measured using a spectrophotometer (Spectro 22, Labo Med, Inc.) at 530 nm (A_{530nm}). Viability was expressed as absorbance at 530 nm per 150 mg.

Experimental design and statistical analysis

All experiments were randomized complete block design (RCBD) with six replicates. The SAS software was used for analysis of variance (ANOVA). Means were compared using the Duncan's multiple range test (DMRT).

RESULTS AND DISCUSSION

Effects of dehydration on survival of non-encapsulated and encapsulated *Dendrobium* sonia-17 PLBs

Moisture content of fresh PLBs was 84% for non-encapsulated and 87% for encapsulated PLBs, with no significant difference obtained between the fresh weights of both groups. The moisture content of non-encapsulated PLBs reduced significantly to 58.7% after 5 h of dehydration (Table 1). However, when the PLBs were encapsulated, the decline in moisture content was not significant despite the 5 h of dehydration treatment (Table 1). Variable results are usually obtained when dehydration

Table 2. Absorbance results for encapsulated and non-encapsulated PLBs of *Dendrobium* sonia-17 that was not subjected to cryopreservation.

Dehydration time	Absorbance (530 nm)	
	Non-encapsulated PLBs	Encapsulated PLBs
0	0.093[b]	0.127[b]
1	0.108[b]	0.150[a]
3	0.006[c]	0.015[c]
5	0.001[c]	0.008[b]

Means with the same letter(s) within a column are not significantly different at $p < 0.05$ based on DMRT.

Table 3. Absorbance data of both cryopreserved non-encapsulated and encapsulated PLBs of *Dendrobium* sonia-17.

Dehydration period	TTC (A_{530})		Regeneration (%)	
	Non-encapsulated PLBs	Encapsulated PLBs	Non-encapsulated PLBs	Encapsulated PLBs
0	1.145[a]	1.145[a]	100.0[a]	100.0[a]
1	1.145[a]	1.145[a]	100.0[a]	100.0[a]
3	0.969[c]	1.130[b]	88.0[b]	98.0[a]
5	0.717[d]	1.101[c]	83.0[b]	98.0[a]

Means with same small letter(s) within a column are not significantly different at $p < 0.05$ based on DMRT.

is performed under the 50 g of oven sterilized silica gel since various factors such as air temperature, relative humidity and airflow rates are inconsistent. More reproducible results could have been obtained if the dehydration had been performed in hermetically-sealed jars containing a specified amount of silica gel (Engelmann et al., 2008).

PLBs in the control experiment (non-cryopreserved) were immediately placed on semi-solid regeneration medium upon reaching the optimal dehydration period (3 h) (Table 2). The results indicated that dehydration affected the regeneration of dehydrated non-encapsulated PLBs, but not the encapsulated PLBs. When fresh PLBs were placed in semi-solid regeneration media, new leaf formation was observed within a week, followed by an increase in both leaf number and size within a period of 21 days. Results based on TTC assay showed that 1-h of dehydration did not affect the survival of non-cryopreserved PLBs but further dehydration resulted in a decline in survival rate (Table 2). However, a high survival (83%) was obtained even after 5 h of dehydration, indicating that the PLBs of *Dendrobium* sonia-17 were tolerant to dehydration (Table 3).

Non-encapsulated PLBs dehydrated for 3 and 5 h were observed to have a lag phase in growth and slow regeneration, indicating a loss in vigor (data not shown). Despite the dehydration treatment, encapsulated PLBs retained their survival, which could be attributed to the minimal loss in moisture content observed after 5 h of dehydration. This result was supported by the TTC absorbance data (A_{530}, Table 2). Both untreated PLBs and freshly-encapsulated PLBs presented an absorbance value of 1.145 (Table 3). The results also indicated a significant decline (Table 2) in the absorbance data as a result of the dehydration. A proper dehydration proved to be vital to assure high survival of cryopreserved plant tissues. The optimal water content of encapsulated tissues varies among plant species or even varieties mainly due to their difference in dehydration tolerance (Wang et al., 2000).

The absorbance values were higher for both dehydrated and freshly-encapsulated PLBs. The protective nature of the capsule and the presence of nutritive substance in the encapsulated media could be contributing factors for the high survival percentage of encapsulated PLBs. However, the survival of both encapsulated and non-encapsulated PLBs were low after cryopreservation (Table 3). This could be attributed to the high moisture content in both treatments. High survival percentages were also observed in the cryopreserved PLBs of *Cleisostoma arietinum,* which were dehydrated until a final water content of approximately 0.63 H_2Og^{-1} dry weights (Maneerattanarungroj et al., 2007).

Effects of sucrose pretreatment (0, 0.3, 0.5 or 0.7M) and dehydration (0, 1, 3 and 5 h) on survival of *Dendrobium* sonia-17 PLBs in liquid nitrogen

Chemicals that promote active osmotic movements such as sugars or sugar alcohols are usually supplemented into media used in cryopreservation experiments (Lambardi et al., 2008; Reed and Uchendu, 2008). Explants placed on media containing sucrose, sorbitol or mannitol under-

Table 4. Mean moisture content (%) and absorbance values in various sucrose pretreatment concentrations and dehydration periods.

Sucrose concentration (M)	Dehydration period (h)	Moisture content	Absorbance (530 nm)	
			-LN	+LN
0	0	86.37	1.612^{d}	0.271^{de}
	1	87.02	1.449^{d}	0.221^{def}
	3	83.12	1.301^{de}	0.112^{ef}
	5	82.46	1.006^{ef}	0.053^{f}
0.3	0	74.10	1.647^{cd}	0.677^{b}
	1	70.96	1.693^{cd}	0.342^{d}
	3	67.50	0.713^{fg}	0.620^{b}
	5	63.95	0.707^{fg}	0.601^{bc}
0.5	0	75.86	2.007^{c}	0.247^{def}
	1	64.44	2.015^{b}	0.393^{cd}
	3	56.76	2.422^{a}	1.032^{a}
	5	55.78	0.544^{g}	0.543^{bc}
0.7	0	68.61	1.999^{c}	0.227^{def}
	1	62.09	1.511^{d}	0.299^{de}
	3	53.15	0.968^{ef}	0.115^{ef}
	5	52.18	0.750^{fg}	0.103^{ef}

Means with same small letter(s) within a column are not significantly different at $p < 0.05$ based on DMRT.

go mild to moderate dehydration stress, which stimulate metabolic changes in the plant cells, and enhances desiccation and freezing tolerance (Lambardi et al., 2008; Reed and Uchendu, 2008). Sugars, which are good glass formers are absorbed by cells during the cryopreservation process and help stabilise the cellular membrane by replacing cellular water and forming hydrogen bonds with the membranous phospholipids (Lambardi et al., 2008, Turner et al., 2001). Sugar-enriched media help increase the production of endogenous cryoprotective compounds such as sugar (sucrose) and sugar alcohols in target explants, which results in cellular membrane stability (Crowe et al., 1984a, b; Jitsuyama et al., 1997; Sakai and Engelmann, 2007). The cells were able to absorb mannitol and produce ABA, proline and stress proteins such as the late embryogenesis proteins (LEA) when preconditioned in 0.3 M mannitol for 24 h (Jitsuyama et al., 1997; Reinhoud, 1997). Excised shoot tips, pretreated for 1 or 2 days on media enriched with 0.3 to 0.7 M sucrose, showed excellent recovery after cryopreservation (Dereuddre et al., 1988; Sakai and Engelmann, 2007; Niino et al., 1992).

The moisture content in beads decreased with the increase of either dehydration period or sucrose concentration in the preculture medium. The moisture content of encapsulated PLBs, placed in 0 M sucrose preculture medium, decreased from 86.4 to 82.5% after 5 h of desiccation. However, the moisture content of the beads decreased from 86.4 to 68.6% when the PLBs were placed in 0.7 M sucrose pretreatment medium for 24 h, without dehydration (Table 4). Sucrose is the most widely used preculture substance as it is non-toxic to cells and has been reported to alleviate or even eliminate harmful effects due to dehydration (Yamada et al., 1991). Survival for unfrozen freshly-encapsulated PLBs (determined based on the TTC absorbance results) was always higher than the cryopreserved samples in all concentrations of sucrose. This indicates that the damage prior to LN exposure was minimal but the freezing process itself was deleterious to the PLBs survival. In cryopreservation of *Dendrobium candidum* Wall ex Lindl, regrowth of none cryopreserved was initiated earlier and preceded faster than the cryopreserved PLBs (Yin and Hong, 2009). Therefore, this result obtained supports previous findings. Survival of cryopreserved PLBs was very low when not treated with sucrose (Table 4). Increased concentrations of sucrose improved PLB post-cryopreservation survival, with 0.5 M sucrose resulting in the highest survival percentage. Therefore, a suitable combination of sucrose pretreatment and dehydration period improved post-thaw survival of cryopreserved PLBs. The best survival percentage was obtained with 0.5 M sucrose pretreatment and 3 h dehydration time was applied in the experiment (Table 4). Encapsulated shoot tips of *Dendrobium* Walter Oumae produced the highest survival ratio and regrowth when pretreated for 2 days in 0.5 M sucrose followed by 6 to 8 h of dehydration under the sterile air flow of the laminar flow hood (Lurswijidjarus

and Thammasiri, 2004).

PLBs precultured on medium containing high sucrose concentrations have been reported to show improved survival after cryopreservation (Ishikawa et al., 1997). Embryogenic cultures, such as protocorm-like bodies, are extremely susceptible to cryoinjuries due to the high water content within the cells. Hence, additional cryoprotection steps such as extra preculture treatments or dehydration are required prior to freezing in liquid nitrogen (Lambardi et al., 2008). It is well established that soluble sugars, heat-stable proteins and dehydrins accumulate during the development of seed and under desiccation stress of vegetative tissues (Black et al., 1999), among other substances such as polyamines, glycerol and proline, which have colligative and non-colligative properties (Panis, 2008).

Conclusion

The best survival percentage in the cryopreservation of the PLBs of *Dendrobium* sonia-17 was obtained when the combination of 0.5 M sucrose pretreatment and 3 h dehydration time was applied in the experiment.

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