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Cryopreservation of *Dendrobium heterocarpum* Lindl. *via* encapsulation-dehydration method

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Abstract. The species *Dendrobium heterocarpum* Lindl., a wild orchid native to north and northeast Thailand, creates an aura of mystique and grandeur with their intricate flower appearance which enthrals many collectors within the country. An increase in collection for trade or any orther purposes may lead to a dramatic decrase in the population of this species, thus becoming rare or endangered species in the near future. Cryopreservation is an alternative means for conservation of plant genetic resources which can be applied to this species. This study, therefore, was aimed to optimize the conditions for encapsulation-dehydration method in *D. heterocarpum*. The protocorms were successfully cryopreserved as the following protocol. The protocorms were encapsulated with Ca-alginate, precultured on solid ND medium supplemented with 0.25 M sucrose for 1 week in the dark at 0°C and subsequently precultured in liquid ND medium supplemented with 0.25 M sucrose for 2 days in the dark at 0°C. Then, they were dehydrated by air-drying for 12 hours and plunged into LN for 1 day. Recovery percentage was 8.33% after 2 weeks of regrowth. However, preculture with ABA was found to insufficiently enhance freezing and thawing tolerance. TTC staining was negative and further regrowth on solid ND medium confirmed no survival of the cryopreserved protocorms.
Key Words: *Dendrobium heterocarpum* Lindl., encapsulation-dehydration, protocorms, cryopreservation, cold acclimation, regrowth, preculture.

Resumen. Las especies de *Dendrobium heterocarpum* Lindl., natural de orquídeas silvestres al norte y noreste de Tailandia, crea un aura de misterio y grandeza con su apariencia de flores intrincado que cautiva a muchos coleccionistas en el país. Esto hace que se convierta en especies raras o en peligro de extinción en un futuro próximo. La crioconservación es un medio alternativo para la conservación de los recursos fitogenéticos que se pueden aplicar a esta especie. Este estudio fue dirigido a optimizar las condiciones para el método de encapsulación-deshidratación en *D. heterocarpum*. El protocormos fueron criopreservados con éxito como el siguiente protocolo. El protocormos fueron encapsulados con Ca-alginato. Eran precultured entonces en un medio sólido ND suplementado con sacarosa 0.25 M durante 1 semana en la oscuridad a 0°C y, posteriormente, en medio precultured ND líquido suplementado con sacarosa 0.25 M durante 2 días en la oscuridad a 0°C. Ellos estaban deshidratados luego por el secado al aire durante 12 horas y se sumergió en nitrógeno líquido durante 1 día. Porcentaje de recuperación fue un 8,33% después de 2 semanas de rebrote. Sin embargo, precultivo con ABA fue encontrado para aumentar suficientemente la congelación y descongelación tolerancia. TTC tinción fue negativa y más rebrote en medio sólido ND confirmó que no existía la supervivencia de la protocormos criopreservados.
Palabras claves: *Dendrobium heterocarpum* Lindl., Encapsulación-Deshidratación, protocormos, criopreservación, Frío al Aclimatación, recrecera, precultura.

Rezumat. Specia *Dendrobium heterocarpum* Lindl., o orhidee sălbatică endemică pentru nordul şi nord-estul Tailandei, creează o aură de mister şi grandoare, iar aspectul complex al florilor captivează mulţi colecţionari din ţară. Datorită acestui lucru specia *Dendrobium heterocarpum* ar putea deveni specie rară sau pe cale de dispariţie în viitorul apropiat. Crioconservarea este o modalitate alternativă pentru conservarea resurselor genetice ale plantelor şi ar putea fi aplicată la această specie. Acest studiu a avut drept scop optimizarea metodelor de încapsulare-deshidratare la *D. heterocarpum*. Protocormii au fost crioconservaţi cu succes după următorul protocol. Protocormii au fost încapsulaţi în alginat de calciu. Au fost apoi precultivaţi pe suport solid ND suplimentat cu zaharoză 0.25 M pentru o săptămâna în întuneric, la 0°C şi ulterior precultivaţi într-un mediu lichid ND suplimentat cu zaharoză 0.25 M pentru două zile la întuneric, la 0°C. Acestea au fost apoi deshidratate prin uscare la aer timp de 12 ore şi cufundate în azot lichid pentru o zi. Procentul de recuperare a fost 8.33% după 2 săptămâni de regenerare. Cu toate acestea, precultura cu ABA nu a fost suficientă pentru a spori toleranţa la îngheţare şi dezgheţare. Colorarea cu TTC a fost negativă şi în continuare regenerarea pe mediu solid ND nu a asigurat supravieţuirea protocormilor crioconservaţi.
Cuvinte cheie: *Dendrobium heterocarpum* Lindl., încapsulare-deshidratatre, protocormi, crioconservare, aclimatizare la frig, regenerare, precultura.

Introduction. Biochemical and physiological mechanisms of low temperature tolerance in higher plants have been intensively studied, leading to comprehending in the mechanisms of plant adaptations to low temperature tolerance. With this understanding, an attempt to preserve explants under ultra low temperature for long-lasting storage termed 'cryopreservation' has been made by developing many useful methods. Up to the present time, there have been many methods e.g. two-step freezing, encapsulation-dehydration, complete vitrification and encapsulation-vitrification developed for different plant parts of many tropical species.

The encapsulation-dehydration method is a process which is based on artificial seed technique by which explants are encapsulated in Ca-alginate beads, and dehydration technique. The dehydration technique enables more flexibility when large numbers of samples are handled as the process is less time consuming than vitrification (Sakai 2000). Moreover, a programmable freezer and high concentration of cryoprotectants are not required, compared to other methods (Reinhoud et al 2000). The encapsulation-dehydration method has been applied to apices (both temperate and tropical species), seeds, somatic embryos and cell suspensions in numbers of species (Engelmann & Takagi 2000). In this method, gradual extraction of water from encapsulated explants is performed during the preculture in sucrose-enrich medium. The sucrose molarities in the beads is further increased by the additional air-drying or desiccation in a laminar air flow cabinet to reach the saturation point of the sucrose solution which results in a glass transition during cooling to -196ºC in liquid nitrogen, preventing ice crystal formation (a cause of lethal damage to living cells) during exposure to ultra low temperature (Engelmann & Takagi 2000). It has been reported that Ca-alginate beads help protect explants during exposure to low temperature. However, dehydration or desiccation has advantages over them. The dehydration technique plays a critical role in survival of the explants by lowering water content, preventing crystalline ice as cited previously. However, the dehydration, in turn, can cause lethal damage to plant cells. Induction of desiccation tolerance through the subsequent increase of sucrose concentration during preculture of encapsulated explants and appropriate duration of exposure are thus the key for the successful cryopreservation.

Numerous factors have been proposed to be involved in adaptation of explants to desiccation and low temperature stress. Sucrose, glucose, fructose and oligosaccharides (e.g. raffinose and stachyose) have been reported as a main factor commonly found in soluble sugars accumulating along with development of freezing tolerance in higher plants, suggesting that these sugars not only serve as osmoprotectants but also play a role in cellular membrane protection from damage caused by dehydration and freezing *via* interaction with lipid bilayer (Ma et al 2009). Apart from sugars, the phytohormone abscisic acid (ABA) has also been proposed to play a role in low temperature and drought tolerance. Cold hardening of plants exhibits an increase in endogenous ABA. This phenomenon implies that ABA is possibly associated with subsequent increase in freezing tolerance of the plants exposed to low temperature (Faltusová-Kadlecová et al 2002). As a result, sugar-, ABA- and cold hardening-based precultures have been widely utilized to enhance desiccation and low temperature tolerance in many plant species.

There have been many relevant studies proposing the suitable conditions for encapsulation-dehydration based cryopreservation in the genus *Dendrobium* which can be applied to *D. heterocarpum*, the specimen used in this study. In this study, therefore, most of the procedures are referred to the literature previously cited, particularly the preculture and dehydration procedures.

Material and Method. Seed-derived protocorms reaching a diameter of 3-4 mm were used as explants. The 3-month old capsules of *D. heterocarpum*, collected from the Agricultural Garden, Department of Horticulture, Faculty of Agriculture, Khon Kaen University, Khon Kaen province were gently washed with detergent and subsequently rinsed in running tap water for 30 min before surface sterilization in a laminar air flow cabinet. Then, they were dipped into 70% (v/v) ethanol for 5 min, subsequently soaked in disinfectant [25%

(v/v) sodium hypochlorite in combination with Tween-20] with continuous shake for approximately 20 min, and repeatedly cleaned with sterile distilled water for 3 times. Sterilization was reaffirmed by dipping the orchid capsules into 70% ethanol and flaming them for 3 times. After that, they were longitudinally dissected, and seeds were then picked off and transferred into liquid new Dogashima (ND) (Tokuhara and Mii, 1993) medium. The cultures were kept at 25 ± 2°C under a long photoperiod (16 h light: 8 h dark) with a photon dose of 40 μmol $m^{-2}s^{-1}$.

Cryopreservation by encapsulation-dehydration method was implemented as the following steps:

Encapsulation, Preculture and Dehydration: The protocorms were immersed in 3% (w/v) Na-alginate prepared in liquid Ca-free ND medium. Each protocorm was dropped in 100 mM $CaCl_2$ and left for 20 min before wash with sterile distilled water for 2 times. In sucrose-preculture, the encapsulated protocorms were precultured on solid ND medium supplemented with 0.25 M sucrose in the dark at 0°C for 1 week and subsequently transferred into liquid ND medium supplemented with 0.25-1.00 M sucrose, kept in the same condition for 2 days. In ABA-preculture, the encapsulated protocorms were transferred into liquid ND medium supplemented with 0-2.5 mg/l ABA and kept at 0°C in the dark for 1 week before dehydration. The precultured beads were dehydrated in a laminar air flow cabinet for 0-12 hours prior to cryopreservation by freezing in liquid nitrogen (LN) for 1 day. Each treatment was repeated 3 times, 10 encapsulated protocorms for each.

Freezing and Thawing: The dehydrated beads were loaded into 1.8 ml cryotubes (10 encapsulated protocorms for each) and directly plunged into LN for 1 day. The cryotubes were then taken out from LN and rapidly warmed in a water bath at 38±2°C for 3 min. The cryopreserved protocorms were then transferred into liquid ND medium supplemented with 0.25 M sucrose for 20 min and washed with sterile distilled water for 3 times before regrowth on solid ND medium.

Water Content: Water content was calculated into g $H_2O \cdot g\ DW^{-1}$ by determining weight loss of the beads dried in an oven at 60°C for 24 hours (Dumet et al 2002).

Electrolyte Leakage: After recovery from LN, the cryopreserved protocorms were loaded in screw vials containing 10 ml deionized water. Then, they were incubated at 32°C for 2 hours and initial electrical conductivity (EC_1) was measured using an electrical conductivity meter (WTW TetraCon®325, InoLab pH/Cond Level1). After that, they were autoclaved at 121°C under the pressure of 1.06 $kg \cdot cm^{-2}$ for 20 min and the final electrical conductivity (EC_2) was measured after they were cooled in a water bath at 32°C. Afterwards, the electrolyte leakage (EL) value was calculated into a percentage using the formula $EL = [EC_1/EC_2] \times 100$.

Viability Test by TTC Staining: Viability was assessed two times: After dehydration and recovery from LN. The cryopreserved beads were loaded in screw vials containing 500 μl TTC (2,3,5- Triphenyltetrazoliumchloride) solution and incubated in the dark at 25°C for 24 hours. The viable protocorms generally show insoluble scarlet formazan because dehydrogenase enzyme in living tissues reduces soluble colorless TTC to insoluble scarlet formazan (Fokar et al1998).

Results and Discussion. Water content of the encapsulated protocorms prior to being subjected to sucrose-preculture (control) was approximately 18.83 g $H_2O \cdot g\ DW^{-1}$. Sucrose preculture dramatically lowered the water content of the encapsulated protocorms, giving rise to low water content of 2.75 g $H_2O \cdot g\ DW^{-1}$ when precultured with 0.25 M sucrose. Further extraction of water from the encapsulated protocorms using sucrose concentrations ranging from 0.25 to 1.00 M gave rise to noticeable results. The precultured beads precultured with sucrose concentrations of 0.50, 0.75 and 1.00 M showed no sign of viability detected by TTC staining. Therefore, only the encapsulated protocorms precultured with 0.25 M sucrose were subjected to dehydration. After dehydration, the water content dropped from 0.14 to 0.07 g $H_2O \cdot g\ DW^{-1}$ (Figure 1c & d; Table 1). Only the cryopreserved protocorms with previous dehydration of 12 hours were viable. The percentage of viability of the cryopreserved protocorms assessed by TTC staining after recovery from LN was 16.67% (Figure 1e & f; Table 1). However, the

recovery percentage dropped to 8.33% after 2 weeks of regrowth on solid ND medium. The EL values among all dehydration times in the preculture group were similar (Table 1). However, the EL values between nonpreculture and preculture groups were significantly different. The EL value of the nonpreculture group was higher than that of the preculture group.

In recent years, vegetative parts e.g. shoot tips, shoot primodials or axillary buds have been widely used as explants to be encapsulated with Ca-alginate in an attempt to produce synthetic seeds since they were considered as more suitable alternatives to somatic embryos which were first proposed by Murashige in 1977 (Sakar & Naik 1997). The synthetic seeds consist of tissues derived from vegetative parts or somatic embryos and artificial endosperms. The tissues were coated with encapsulation matrix (Ca-alginate), subsequently forming artificial endosperm. The complexing agent was formed after Na-alginate was mixed with $CaCl_2$ with di- and trivalent metal cations to form Ca-alginate through formation of ionic linkages between carboxylic acid groups on the guluronic acid molecules of the alginate (Redenbaugh et al 1993). Polyethyleneimine (Kersulec et al 1993), chitosan (Tay et al 1993), potassium alginate, gelatin, carrageenan, agar, agarose, gelrite, carboxymethyl cellulose, glycerol monooleate, paraffin and polyacrylamide have also been used as encapsulation matrix (Redenbaugh et al 1993; Datta et al 1999). The condition of an artificial endosperm could provide nutrients for plantlet development from encapsulated tissues (Nieves et al 1998). Among all types of encapsulation matrices, Na-alginate has been widely used because it solubilises and remains stable at ambient temperature. Besides, it does not require heat to produce gel, but immediately begins forming encapsulation matrix when brought into contact with metal cations (Redenbaugh et al 1993).

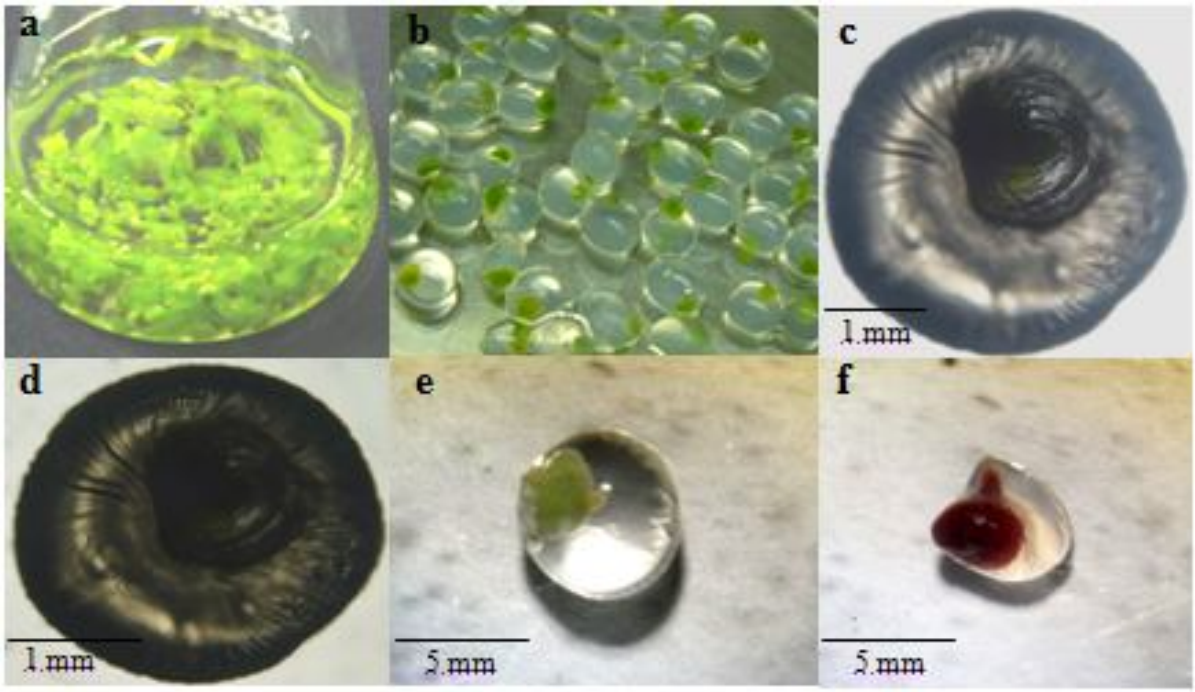

Figure 1. Explants, artificial seeds and viability test of cryopreserved artificial seeds: a) Protocorms as explants for producing artificial seeds, b) Artificial seeds, c) Artificial seeds after dehydration without previous sucrose-preculture, d) Artificial seeds after dehydration with previous sucrose-preculture, e & f) Soluble colourless compound and insoluble scarlet formazan in cryopreserved artificial seeds tested by TTC staining indicating that cells were non-viable and viable, respectively.

In this study, the seed-derived protocorms were used as explants (Figure 1a), and Na-alginate and $CaCl_2$ were used as complexing agents to produce artificial seeds (Figure 1b) because of their advantages cited previously. The results showed high possibility of artificial seed

production in *D. heterocarpum* by which the seed-derived protocorms were used as explants coated with encapsulation matrix, Ca-alginate. The artificial seeds performed high potential of seed germination (100%) when regerminated on solid ND medium. The encapsulated protocorms successfully developed into whole plantlets by undergoing shoot formation penetrating out of the encapsulation matrix followed by root formation. Their growth and development rate was slightly slower than that of naked protocorms. This indicated that the encapsulation matrix involved in retarding germination of the artificial seeds. Light was found to play an important role in stimulating rapid growth and development of the encapsulated protocorms. Therefore, they were usually kept in the dark condition to avoid invidious shoot and root formation as this would result in depletion of artificial endosperm, leading to undesirable short-term storage.

Although the artificial seed technique is considered easy to handle using a dropper, pipette, syringe or spatula; the size of the artificial seeds as well as the position of the protocorms within the encapsulation matrix produced by different equipments may vary. Dropper, pipette and syringe were suitable for small size of explants e.g. seeds, whereas spatula was appropriated for larger explants e.g. protocorm-like bodies (PLBs), shoot tips and nodal explants. In this experiment, spatula was used to produce the artificial seeds. The results showed that the size of the artificial seeds varied ranging from a diameter of 5 to 8 mm, and the position of the protocorms were usually found on either side of the encapsulation matrix. Some were found to be half embedded. Therefore, acquisition was required for this work.

Table 1

Effect of dehydration time on water content, electrolyte leakage, viability and recovery percentages of cryopreserved artificial seeds

Dehydration time (h)	*Water content (g $H_2O \cdot g\ DW^{-1}$)^Z*	*Electrolyte leakage (%)^Z*	*Viability (%)^Z*	*Recovery (%)^Z*
0^a	18.83±0.36^a	86.52±7.08^a	0±0.00^a	0±0.00^a
0^b	2.75±0.51^b	70.78±5.76^b	0±0.00^a	0±0.00^a
8	0.14±0.01^c	76.38±3.16^b	0±0.00^a	0±0.00^a
9	0.13±0.02^c	73.96±3.84^b	0±0.00^a	0±0.00^a
10	0.12±0.03^c	73.23±8.11^b	0±0.00^a	0±0.00^a
11	0.09±0.02^c	69.21±3.87^b	0±0.00^a	0±0.00^a
12	0.07±0.02^c	71.23±6.12^b	16.67±0.00^b	8.33±0.00^b

0^a – Without sucrose-preculture, 0^b – with sucrose-preculture, ^{Z} – data is the means of five replicates and means with the same alphabet (a, b or c) are not significantly different at p=0.05 using Duncan's multiple range test.

Cryopreservation has become important as a means of ensuring the long-term preservation of plant germplasms (Kami et al 2009). Cryopreservation of biological tissues can be useful only if intracellular ice crystal formation is avoided since this causes irreversible damage to cell membranes, thus destroying their semi-permeability. In nature, some plants adopted systems where ice crystal formation at sub-zero temperatures can be avoided through the synthesis of specific substances (e.g. sugars, proline and proteins) that lower the freezing point in living plant cells, resulting in 'supercooling'. Such avoidance of crystallization requires well preparation of explants prior to exposure to ultra-low temperature, while still maintaining a minimal moisture level needed to maintain viability. The cell cytosol can be concentrated to lower cellular water through air drying, freezing dehydration, application of penetrating or nonpenetrating substances (cryoprotectants), or adaptive metabolism (hardening) (Panis & Lambardi 2005). Membranes are often damaged during the process of freezing and thawing, or during desiccation or dehydration. Rupture of plasma membrane is one of the most commonly used indicators of cell death (Wolfe & Bryant 1999). Various methods e.g. vitrification, encapsulation-dehydration and encapsulation-vitrification have been developed since the second half of the 1980s (Kami et al 2009). Among all

the developed methods cited previously, the encapsulation-dehydration method was selected to be applied to *D. heterocarpum* as it is a simple method involved in application of sugars, dehydration, cold acclimation and/or ABA. In many laboratories, of all sugars, sucrose is usually utilised as it is inexpensive.

The application of exogenous ABA for triggering endogenous ABA synthesis may be excluded as the endogenous ABA synthesis can be alternatively induced by cold acclimation alone or in combination with sucrose-preculture. The cold acclimation is always used to enhance cold hardening or cold tolerance for it is associated with multiple mechanisms that include changes in gene expression, membrane composition and cryobehavior, antioxidants and accumulation of substantial amount of compatible solutes e.g. soluble sugars, amino acids and glycine betaine which are proposed to play a role in protection of cells from freezing injury (Ma et al 2009).

In this study, the application of sucrose in combination with cold acclimation, and the application of ABA alone were included. Numerous studies have demonstrated that the application of exogenous ABA enhanced dehydration and/or freezing tolerance through ABA-responsive genes and ABA-responsive proteins (Bian et al 2002). The application of exogenous ABA has been reported to induce the accumulation of sugars, heat-stable proteins and dehydrins. The dehydrins and other heat-stable proteins were not detectable after removal of the exogenous ABA but reappeared when PLBs were desiccated to reach the water content below 1.0 g $H_2O \cdot g\ DW^{-1}$. An increase in the accumulation of soluble sugars in ABA-treated samples was associated with an augmentation of dehydration tolerance in PLBs at water content of 0.5 g $H_2O \cdot g\ DW^{-1}$. However, there was no significant effect on the freezing tolerance or survival rate after freezing (Bian et al 2002). This was consistent to the study reporting that ABA-treated protocorms improved desiccation tolerance, but failed to develop freezing and thawing tolerance despite the fact that ABA-treated protocorms performed low water content in the range of unfrozen state in which water content was less than 0.25 g $H_2O \cdot g\ DW^{-1}$ (Volk & Walters 2006). The failure in development of freezing tolerance may result from the short-time remaining of stress-responsive proteins induced by ABA, leading to failure in development of freezing tolerance. This was consistent to the study demonstrating that ABA-induced proteins remained no longer than 1 day (Bian et al 2002). The application of sucrose in combination with ABA in the preculture process, in turn, showed that it promoted viability after desiccation, freezing and thawing. The capacity to survive when stored in LN was also dependent upon genotype and type of cells (Ashmore 1997; Lipavska & Vreugdenhil 1996).

Explant type and physiological stage could influence the duration of storage due to the fact that type and nature of cells determined the ability of cells to withstand freezing stress (Ashmore 1997; Engelmann 1997; Swan et al 1999). In general, explants from rapidly growing cultures were recommended to be used for cryopreservation since actively dividing cells had dense cytoplasm and little developed vacuolar system which made them more likely to withstand freezing and remained viable (Ashmore 1997; Moges et al 2003). The failure in freezing tolerance in this study may result from lack of subculture of the protocorms used, not only preventing protocorm proliferation but leading to production and accumulation of numerous metabolites and vacuolar systems during elongation and ramification to form a multiple branched structure prior development into whole plantlets. This directly affected successful drainage of water out of the cells, but this was not the case as the amount of water recorded from ABA-treated protocorms revealed low water content in the range of unfrozen state as cited previously. Conversely, it might be the thickness of the late protocorms which prevented penetration of sufficient amount of exogenous ABA into the cells to induce sufficient amount of endogenous ABA synthesis involving in accumulation of soluble sugars, stress-responsive proteins and dehydrins to enhance freezing and thawing tolerance.

However, the application of sucrose in combination with cold acclimation enhanced freezing tolerance in late protocorms used when dehydrated for 12 hours. This was consistent to the study reporting that the inclusion of ABA to sucrose-enriched cryoprotective medium prior to cryopreservation influences post-thaw viability by enhancing desiccation tolerance (Danso & Ford-Llyod 2008). Although ABA was not added

to the sucrose-enriched medium in this study, cold acclimation alternatively performed as ABA addition as cited previously that cold acclimation triggered endogenous ABA synthesis.

Conclusions. The study provided the optimal conditions for both protocorm induction and cryopreservation of *D. heterocarpum*. The ND medium greatly enhanced rapid germination and protocorm induction, thus being considered as a suitable medium for both seed germination, and protocorm induction and proliferation although plant growth regulators were excluded. Successful cryopreservation was performed by preculturing the encapsulated protocorms on solid ND medium supplemented with 0.25 M sucrose and kept at 0ºC in the dark condition for 1 week. Sucrose molarity in the precultured beads was further concentrated by repeatedly preculturing the beads in liquid ND medium supplemented with 0.25 M sucrose for 2 days prior to dehydration for12 hours.

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