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# Long-term preservation of *Dendrobium chrysotoxum* Lindl. using encapsulation method

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**Abstract**. The species *Dendrobium chrysotoxum* Lindl., a wild orchid native to north and northeast Thailand, is rapidly depopulated in its natural stand by ingathering for trade within the country. This causes it to become a rare or endangered species in the near future. Long-term preservation is an alternative approach for conservation of plant genetic resources which can be applied to this species. This study was aimed to optimize the conditions for seed germination, protocorm induction and proliferation, and preserving *D. chrysotoxum* protocorms via encapsulation-dehydration method. Seeds successfully germinated on solid ND medium in the absence of plant growth regulators. Solid ND medium supplemented with 4 mg/l BA was found to promote protocorm proliferation. The proliferation rate was 85%. The seed-derived protocorms were successfully cryopreserved as the following protocol. The protocorms were encapsulated with Ca-alginate and then precultured in liquid ND medium supplemented with 0.25 M sucrose for 1 week in the dark at 4 °C. They were further precultured in liquid ND medium supplemented with 0.75 M sucrose for 2 days in the dark at 4 °C and dehydrated by air-drying for 5 h and subsequently placed in a freezer at -80 °C for 2 weeks. TTC staining exhibited viability rate of 64%.
**Key Words**: *Dendrobium chrysotoxum* Lindl., encapsulation-dehydration, protocorms, long-term preservation, preculture.

**Rezumat**. Specia *Dendrobium chrysotoxum* Lindl., o orhidee sălbatică nativă în nordul şi nord-estul Tailandei, este supusă unei depopulări rapide în arealul său natural din cauza recoltării ei în scopuri comerciale. Acesta face ca planta să devină o specie rară sau periclitată în viitorul apropiat. Prezervarea pe termen lung reprezintă o abordare alternativă pentru conservarea resurselor genetice vegetale care poate fi aplicată la această specie. Scopul acestui studiu a constat în optimizarea condiţiilor de germinare a seminţelor, induccerea şi proliferarea protocormilor şi conservarea protocormilor de *D. chrysotoxum* prin metoda de deshidratare-încapsulare. Seminţele au germinat cu succes pe mediu ND solid în absenţa factorilor vegetali de creştere. S-a observat că mediul ND solid suplimentat cu 4 mg/l BA stimulează proliferarea protocormilor. Rata de proliferare a fost de 85%. Seminţele rezultate din protocormi au fost crioprezervate cu succes prin protocolul descris mai jos. Protocormii au fost încapsulaţi cu alginat de Ca şi apoi precultivat în mediu ND lichid suplimentat cu 0.25 M zaharoză pentru o săptămână la întuneric şi at 4 °C. Au fost apoi precultivaţi în mediu ND lichid suplimentat cu zaharoză 0.75 M pentru 2 zile şi la 4 °C şi deshidratate prin aerare timp de 5 ore şi apoi au fost plasate la -80 °C pentru 2 săptămâni. Prin colorare cu TTC s-a determinat o viabilitate de 64%.
**Cuvinte cheie**: *Dendrobium chrysotoxum* Lindl., încapsulare-deshidratare, protocormi, crioconservare, precultură.

**Introduction**. The species *Dendrobium chrysotoxum* Lindl., a wild orchid native to north and northeast Thailand, is an epiphytic orchid that is likely to be depopulated in its natural environment because it is increasingly collected for trade within the country. With the understanding of biochemical and physiological mechanisms of low temperature tolerance in higher plants, there is an attempt to preserve explants under ultra low temperature for long-term storage termed 'cryopreservation'. Many approaches e.g. two-step freezing, encapsulation-dehydration, complete vitrification and encapsulation-vitrification have been developed. Among these approaches, the encapsulation-dehydration method is widely employed as it is less time consuming than any other methods (Sakai 2000). Moreover, a programmable freezer and high concentration of cryoprotectants as compared to other methods are not required (Reinhoud et al 2000). In this method, gradual extraction of water from explants is performed during the sucrose

preculture. The sucrose molarities in the beads is further increased by the additional air-drying in a laminar air flow cabinet to reach the saturation point of the sucrose solution resulting in a glass transition during cooling to ultra low temperature preventing ice crystal formation, causing living cells to be injured, during exposure to that condition (Engelmann & Takagi 2000). It has also been reported that Ca-alginate beads help protect explants during exposure to low temperature. The dehydration technique plays a critical role in survival of the explants by lowering water content, preventing crystalline ice. However, 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.

Sugars, abscisic acid (ABA) or cold hardening have been proposed involving in adaptation of explants to desiccation and low temperature stress. Sugars have been reported as a key 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). 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, indicating that ABA is possibly associated with subsequent increase in freezing tolerance of the plants exposed to low temperature (Faltusová-Kadlecová et al 2002). Thus, precultures with sugar, ABA or cold hardening have been widely employed to enhance desiccation and low temperature tolerance in many plant species. This study, therefore, was aimed to optimize the conditions for long-term preservation of *D. chrysotoxum* via encapsulation-dehydration method, regarding sucrose concentrations used, duration of sucrose preculture and duration of cold hardening.

**Material and Method**. Protocorms reaching a diameter of 5 mm were used as explants. The 3-month old pods of *D. chrysotoxum*, ingathered after artificial pollination at the Department of Biology, Faculty of Science, Khon Kaen University, Khon Kaen province, were gently washed with detergent and subsequently rinsed in running tap water for 30 min before surface sterilization. They were then 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 cleaned with sterile distilled water for 3 times. Sterilization was repeated 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) medium (Tokuhara and Mii, 1993). For protocorm induction, protocorms were cultured on solid ND medium supplemented with various concentrations of naphthalene acetic acid (NAA) and benzylamine (BA). 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 $m^{-2}s^{-1}$.

Long-term preservation via encapsulation-dehydration method was performed as follows.

*Encapsulation, preculture and dehydration*

The protocorms were immersed in 3% (w/v) Na-alginate prepared in liquid ND medium. Each protocorm was dropped in 100 mM $CaCl_2$ and left for 20 min before washing with sterile distilled water for 3 times. In sucrose preculture, the artificial seeds were precultured in liquid ND medium supplemented with 0.25 M sucrose in the dark at 4 °C for 1 week and subsequently transferred to liquid ND medium supplemented with 0.75 M sucrose for 2 d. Thereafter, they were dehydrated in a laminar air-flow cabinet for 1-5 h prior to freezing.

*Freezing and Thawing*

The dehydrated beads were loaded into 1.8 ml cryotubes (10 encapsulated protocorms for each) and directly placed in a freezer at -80 °C for 2 weeks. The cryotubes were then taken out from a freezer 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).

*Viability Test by TTC Staining*

Viability was assessed two times, after dehydration and recovery from freezing. 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 al 1998).

**Results and Discussion**. Seeds successfully germinated and developed into protocorms and plantlets on solid ND medium in the absence of plant growth regulators. Protocorms profoundly proliferated with a proliferation rate of 85% when cultured on solid ND medium supplemented with 4 mg/l BA (Figure 1), while the protocorms cultured on the same medium in the absence of plant growth regulators continue differentiation and ramifying to form shoots and roots. In this study showed that BA alone was sufficient to promote protocorm induction and proliferation. This was consistent to the study by Kong et al (2007) reporting that addition of 0.5 mg/l 6-BA alone enhanced protocorm proliferation in *Dendrobium strongylanthum*. This could be inferred that plant growth regulators are a key factor to induce or hinder development into protocorms or plantlets in many higher plant species.

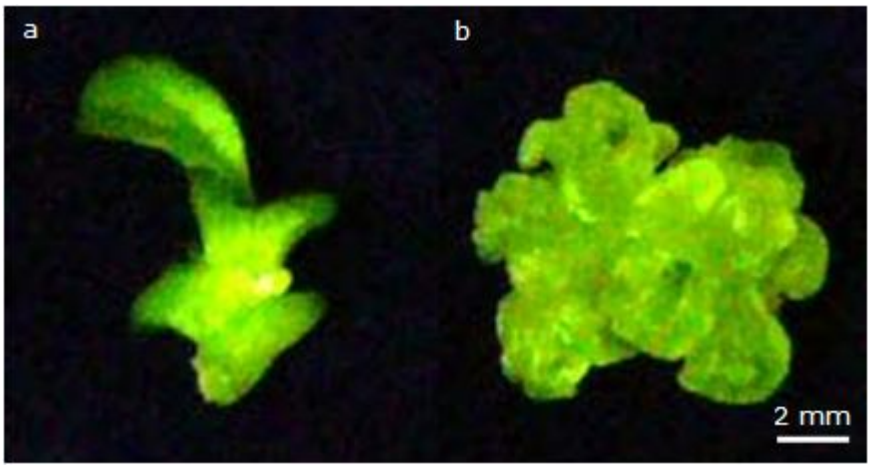


Figure 1. Protocorm proliferation: a) control, b) protocorms cultured on solid ND medium supplemented with 4 mg/l BA.

Growth and development of the protocorms within artificial seeds was shown in Figure 2. The protocorms gradually expanded and differentiated to form shoots or roots to penetrate the encapsulation matrix and further ramify to form a multi-branch structure. Compared to control (naked protocorms), the encapsulated protocorms have a slower rate of growth and development. This may result from encapsulation matrix that hindered them from differentiating to produce shoots or roots to penetrate the matrix.

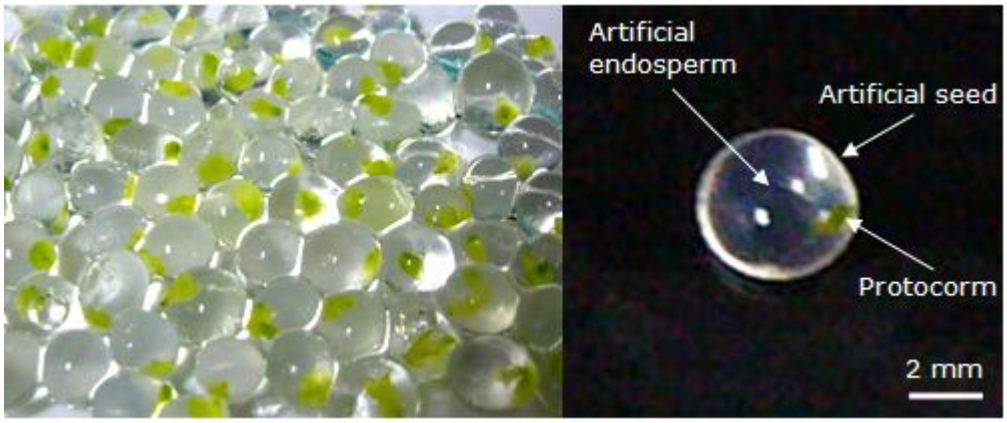

Figure 2. Protocorm proliferation within artificial seeds of *D. chrysotoxum*.

Water content of artificial seeds gradually dropped when precultured wih sucrose while dehydration by air-drying in a laminar air flow cabinet dramitically lowered water content. Protocorm viability was maintained after dehydration without freezing in a freezer. Howver, viability rated gradually dropped after freezing at -80 °C for 2 weeks in a freezer (Table 1 & Figure 3). This indicated that water content in artificial seeds plays an important role in viability of the protocorms. It has been reported that low water content in the range of unfrozen state prevent formation of crystalline ice, causing lethal damage to plant cells (Volk & Walters 2006). Moreover, numerous studies have demonstrated that application of exogenous ABA enhanced dehydration and/or freezing tolerance through ABA-responsive genes and ABA-responsive proteins (Bian et al 2002). The 2 studies cited previously supported this study that utilized sucrose preculture to extract water from artificial together with application of cold acclimation to induce endogenous ABA production to enhance freezing or dehydration tolerance in *D. chrysotoxum*.

Table 1

Water content of artificial seeds after dehydrated for 0-5 h and protocorm viability after subsequent freezing at -80 °C for 2 weeks in a freezer

| *Dehydration time (h)* | *Water content*[z] | *Protocorm viability (%)*[z] |
|---|---|---|
| 0 | 27.81±0.3[a] | 0±0.0 [a] |
| 1 | 2.5±1.02[b] | 17±0.8 [b] |
| 2 | 2.1±0.07[c] | 22±1.7 [c] |
| 3 | 1.7±0.08[d] | 34±3.1 [d] |
| 4 | 1.1±1.01[e] | 47±2.4 [e] |
| 5 | 0.3±0.02[f] | 64±1.2 [f] |

[z] – data were analyzed using one way ANOVA and the differences contrasted using Duncan's multiple range test - different letters indicate values are significantly different ($P<0.05$).

Furthermore, there was a study proposing that exogenous ABA was generally used to induce the accumulation of sugars, heat-stable proteins and dehydrins. The dehydrins and other heat-stable proteins were not detectable after removal of exogenous ABA but reappeared when PLBs were desiccated to water contents below 1.0 g $H_2O \cdot g\ DW^{-1}$. The increased accumulation of soluble sugars in ABA treated samples was associated with increased dehydration tolerance of 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 rated after freezing (Bian et al 2002). Application of exogenous ABA may result in failure in development of freezing tolerance which may result from stress-responsive proteins induced by ABA remained in a very short time leading to failure in development of freezing tolerance. This was supported by Bian et al (2002) that ABA-induced proteins remained no longer than 1 day.

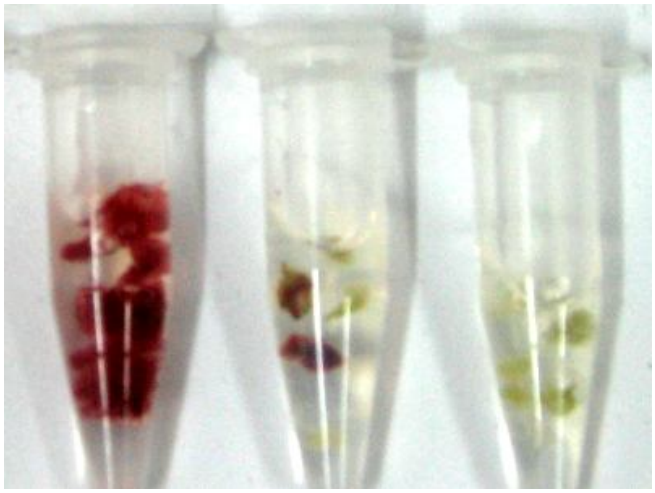

Figure 3. Viability test by TTC staining.

Also, it was reported that a lower water content in plant material prevents nitrification of cytoplasm during freezing (Niino and Sakai 1992). Protocorms in alginate beads that were not sufficiently dried could not ably survive. This was caused by a large amount of crystalline ice in the cytoplasm.

Therefore, it is crucial to optimize the conditions for long-term storage in each species regarding the method used.

**Conclusions**. Seed germination and development into protocorms or plantlets are dependent upon numerous factors. Medium type play an important role in a successful seed germination of an orhcid while plant growth regulators have a little effect on seed germination. However, plant growth regulators have a profound influence on protocorm proliferation and development into plantlets. In this study, it is suggested that seeds be cultured on solid ND medium in the absence of plant growth regulators to yield a successful seed germination. Moreover, it is required that protocorms be cultured on the same medium supplemented with 4 mg/l BA to enhance protocorm proliferation. Apart from seed culture, this study also provided a feasible long-term preservation of *D. chrysotoxum* under sub-zero temperature (-80 ºC). The study suggested the optimal condition as follows. The protocorms were encapsulated with Ca-alginate and then precultured in liquid ND medium supplemented with 0.25 M sucrose for 1 week in the dark at 4 °C. They were further precultured in liquid ND medium supplemented with 0.75 M sucrose for 2 d in the dark at 4 °C and dehydrated by air-drying for 5 h and subsequently placed in a freezer at -80 °C for 2 weeks. This yield a viability rate of 64%.

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