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Original Article

Cryopreservation of *Cymbidium finlaysonianum* Lindl. by encapsulation-dehydration method

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Abstract

Thailand is home to a variety of wild orchids, most of which grow on trees in the forests. However, overexploitation of wild populations along with loss of habitat has accounted for the threatened status of wild orchids. Hence, conservation of wild orchids is urgently needed in the country. The specific purpose of this study was to establish a simple and reproducible protocol for the encapsulation-dehydration based cryopreservation of the orchid species *Cymbidium finlaysonianum*, which represents one of the threatened orchid species in Thailand. In this study, protocorms (2-3 mm in diameter) of the orchid were precultured for 24 h in liquid New Dogashima (ND) medium containing 0-1 M sucrose, encapsulated in 3% w/v Ca-alginate, pretreated for 24 h in liquid ND medium supplemented with 1.5 M sucrose and dehydrated for 0-12 h before plunging into liquid nitrogen for 48 h. The results showed that preculture of the protocorms with 0.75 M sucrose before encapsulation, followed by pretreatment with 1.5 M sucrose and dehydration for 12 h yielded the highest viability and regrowth (65.56%) of the protocorms determined following a 48 h cryopreservation process. Random amplified polymorphic DNA analysis employed for comparison of the polymorphic bands between plantlets raised from non-cryopreserved and cryopreserved protocorms revealed a similar index value of 0.998 indicating the genetic stability of the cryopreserved protocorms.

Keywords: encapsulation-dehydration technique, protocorms, RAPD analysis, cryopreservation

1. Introduction

Thailand is well-known as one of the leading growers and exporters of orchid cut-flowers and potted orchids, as well as home to numerous wild orchids. However, with the increased demand for Thai native orchids, owing to their unique characteristics and fragrance, immense quantities of wild orchids have been collected within the country every year leading to a rapid decline in their populations with a tendency to be at risk of extinction. Apart from overexploitation of wild populations, Thai wild orchids have long been threatened by the extension of agricultural areas and shifting cultivation, as well as cumulative environments. Among the potential threats, orchid smugglers have long been and are still the most serious one to epiphytic wild orchids, as evidenced by the fact that Belgian authorities launched Operation Nero Wolfe in 1990, in which they seized thousands of smuggled orchids from Thailand. Moreover, the Department of Forestry of Thailand recently estimated that nearly six hundred thousand wild orchids are illegally exported each year, mainly to Japan and Europe (Orlean, 1998). Presently, the majority of Thai native orchids are recorded in the red list of species by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), indicating the threatened status of the orchids.

Nowadays, both *in-situ* and *ex-situ* conservations have been used to preserve many orchid species. *In-situ* conservation is defined as conservation of ecosystems and natural

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habitats, the maintenance of viable populations of the species within their natural surroundings, and, in the case of cultivated species, in the surroundings where they have developed their distinctive properties. *Ex-situ* conservation, on the other hand, refers to a technique of conservation of biological diversity outside its natural habitats, targeting all levels of biodiversity such as genetics, species, and ecosystems by means of propagation, micropropagation or *in-vitro* culture (Hirano *et al.,* 2005). Cryopreservation represents one of successful *ex-situ* conservation methods with over 200 plant species conserved by this method (Gámez-Pastrana *et al.,* 2011). This technology is based on the long-term storage of plant materials at low and ultra-low temperatures, usually in liquid nitrogen at -196 °C.

Many different cryopreservation methods have been proposed for successful conservation of Thai orchids, including vitrification (Thammasiri, 2000; Thammasiri & Soamkul, 2007), encapsulation-dehydration (Hongthongkham & Bunnag, 2014; Maneerattanarungroj *et al.,* 2007) and encapsulation-vitrification (Thammasiri, 2008), with plenty of protocols established for the genus *Dendrobium* (Bunnag *et al.,* 2009; Pimda & Bunnag, 2010; Thammasiri, 2008). Unfortunately, only a few studies have been implemented to establish simple and reliable protocols for the genus *Cymbidium*. Establishing a promising protocol for cryopreservation of the members of this genus is of great importance. The species *Cymbidium finlaysonianum* is native to the central, north-eastern regions, south-eastern regions, and the peninsula of Thailand (Cribb & Pedersen, 2014). It is currently arranged in Appendix II in the Checklist of CITES species and is in particular on the verge of extinction due to habitat destruction and indiscriminate harvesting (Islam *et al.,* 2015). Hence, cryopreservation of this orchid species is urgently needed.

Among available cryopreservation techniques, encapsulation-dehydration has received much attention in recent years because of its simple, easy-to-handle and cost-effective procedures which allow for high genetic stability of cryopreserved materials. Moreover, this technique causes no toxicity and is even applicable to dehydration-sensitive explants (Popova *et al.,* 2016; Zainuddin *et al.,* 2011). More importantly, this technology is suitable for cryopreservation of plant protocorms. Therefore, cryopreservation of the *C. finlaysonianum* protocorms using encapsulation-dehydration method is of particular interest.

For this purpose, this study was carried out to establish a simple and reproducible protocol for the encapsulation-dehydration based cryopreservation of *C. finlaysonianum* protocorms. In this context, sucrose concentrations in the preculture step and the length of time required for dehydration of explants were optimized in order to yield high viability and regrowth of the orchid protocorms following cryopreservation in liquid nitrogen. Random amplified poly-morphic DNA (RAPD) analysis was also performed to detect the genetic stability of plantlets raised from cryopreserved protocorms.

2. Materials and methods

2.1 Plant materials and culture conditions

Seven-month-old capsules of *C. finlaysonianum* were collected from Tham Pha Nam Thip Non-Hunting Area, Bueng Ngam Sub-district, Nong Phok District, Roi Et Province, Thailand. The orchid capsules were surface-sterilized in 70% (v/v) ethyl alcohol for 5 min, followed by soaking in 95% (v/v) ethyl alcohol. After flaming off the excess alcohol the capsules were dissected, and the seeds were scraped out and cultured on solid modified New Dogashima (ND) medium (Tokuhara & Mii, 1993). The medium contained 10% (w/v) potato extract, 1% (w/v) sucrose, and 1% (w/v) agar. The pH of the medium was adjusted to 5.4 before sterilization by autoclaving at 121 ºC for 20 min. The cultures were kept at 25 ± 2 ºC under a long photoperiod (16-h light/8-h dark cycle) with a photon dose of 40 µmol/m^2/s. The seeds successfully developed into protocorms after 4 weeks of germination on solid modified ND medium supplemented with 1.5 mg/L kinetin. The obtained protocorms were further cultured for 8 weeks on the same medium with subculture periods at 4-week intervals, and healthy plantlets were achieved. For cryopreservation purposes, protocorms (size 2-3 mm in diameter) were used.

2.2 Cryopreservation experiments

2.2.1 Preculture, encapsulation, and pretreatment

Two-month-old protocorms (size 2-3 mm in diameter) were precultured in liquid modified ND medium supplemented with various sucrose concentrations (0, 0.25, 0.50, 0.75, and 1 M) for 24 h. The precultured protocorms were then immersed in 3% (w/v) Ca-alginate solution and synthetic seeds were formed in 100 mM $CaCl_2$ solution all of which were prepared in liquid modified ND medium. After settlement for 30 min, alginate beads were washed three times with sterile distilled water. The obtained beads were then pretreated with 1.5 M sucrose solution prepared in liquid modified ND medium for 24 h before the dehydration step. Dehydration of the pretreated synthetic seeds was performed by means of air-drying in a laminar air-flow cabinet for 0, 3, 6, 9, and 12 h prior to a 48-h cryopreservation process in liquid nitrogen. Alginate beads not subjected to the preculture and pretreatment steps served as Control I, while those subjected to pretreatment with 1.5 M sucrose acted as Control II. For measurement of water content, the dehydrated beads from each treatment were incubated at 60 ºC for 24 h and the water content was calculated based on the weight loss of the dried beads using the equation: water content (in g H_2O/g DW) = (FW - DW)/DW, where FW and DW represent the fresh weight and dry weight of the beads, respectively.

2.2.2 Freezing, thawing, and regrowth

Following the dehydration step, 15 dehydrated beads from each treatment were transferred to a 1.8 mL cryotube and the cryotubes were directly immersed into liquid nitrogen for 48 h. At a given time point, the cryotubes were removed from the liquid nitrogen and rapidly thawed in a water bath at 40 ºC for 2 min. The cryopreserved protocorms were preliminarily analyzed for their viability by 2,3,5-triphenyltetrazolium chloride (TTC) staining or tetrazolium test. The 15 cryopreserved protocorms from each treatment were transferred to a tube. After addition of 1 ml of TTC

solution, the tubes were incubated in the dark at 37 ºC for 24 h. Assessment of the regrowth ability of the cryopreserved protocorms was carried out on solid modified ND medium. Regrowth capacity of the cryopreserved protocorms was determined based on their ability to survive and develop into whole plants following a 4-week regrowth process.

2.3 DNA extraction and RAPD analysis

Young leaves (0.25 g fresh weight) of 6-month-old orchid plants raised from non-cryopreserved and cryopreserved protocorms were ground in liquid nitrogen using mortar and pestle, and subjected to genomic DNA extraction according to the protocol of (Doyle & Doyle, 1987). The contents of purified DNA were measured using a NanoDrop spectrophotometer. Isolated high quality DNA was diluted to the required concentrations for further use.

For RAPD genotyping, 45 different 10-mer primers of RAPD markers (Operon Technologies Inc., USA) were used in this study. DNA amplifications were carried out in a final reaction volume of 20 µl containing 10X PCR buffer, 25mM $MgCl_2$, 10 mM dNTPs, primer, Taq DNA polymerase and 10 ng/µl of genomic DNA in a 96-well Thermo Hybaid Thermal Cycler. Each amplification profile consisted of 35 cycles: 4 min at 94 ºC, 30 sec at 94 ºC, 40 sec at 45 ºC, 1 min at 72 ºC, and 5 min at 72 ºC. PCR products were separated by electrophoresis on a 1.5% agarose gel and viewed under UV illumination following staining with ethidium bromide.

For data analysis, PCR fragments of RAPD with the same molecular weight were scored as identical, and the presence (1) or absence (0) of amplified DNA fragments was determined for each treatment. Only clear, unambiguous and reproducible RAPD markers were used for the analysis. An evaluation of the similarity index (SI) was conducted according to the formula of Nei and Li (1979).

2.4 Statistical analysis

A completely randomized design with three replicates and 15 protocorms per replicate was used to determine the effect of preculture/pretreatment and dehydration time on viability, regrowth, and genetic stability of the cryopreserved protocorms. The results are expressed as mean ± one standard error (SE) of three replicates and data were analyzed using Scheirer-Ray-Hare test with Duncan's multiple range test to determine the significance relative to the control. In all cases, $P < 0.05$ was considered significant.

3. Results

3.1 Water contents, viability, and regrowth of cryopreserved protocorms

Table 1 shows the water content of encapsulated protocorms subjected to preculture with various sucrose concentration followed by pretreatment with 1.5 M sucrose prior to dehydration for 0-12 h. The results revealed a significant decrease in the water content of encapsulated protocorms with increased sucrose concentrations in the preculture step. Likewise, extending the dehydration time also caused a profound reduction in the water content. Without preculture/pretreatment and dehydration, encapsulated protocorms showed the highest water content of 4.87 g H_2O/g DW. Encapsulated protocorms subjected to preculture with 0-1 M sucrose followed by pretreatment with 1.5 M sucrose also exhibited high levels (1.19-2.80 g H_2O/g DW) of water content without dehydration. Preculture/pretreatment of encapsulated protocorms with subsequent dehydration caused a significant decrease in the water content. Low water content was observed for those subjected to preculture with 0-1 M sucrose, pretreatment with 1.5 M sucrose, and dehydration for 9-12 h. The lowest water content of 0.08 g H_2O/g DW was observed for encapsulated protocorms that were dehydrated for 12 h without preculture and pretreatment.

Table 1. Effect of sucrose preculture/pretreatment and dehydration time on water contents of the encapsulated *C. finlaysonianum* protocorms.

Dehydration Time (h)	Water Content (g H_2O/ g DW) ±SE						
	Controls		Concentrations of Sucrose				
	1	2	0M	0.25M	0.5M	0.75M	1M
0	4.87 ± 0.08^{Aa}	2.80 ± 0.05^{Ab}	1.69 ± 0.01^{Ac}	1.63 ± 0.03^{Ad}	1.62 ± 0.02^{Ae}	1.50 ± 0.02^{Af}	1.19 ± 0.07^{Ag}
3	3.31 ± 0.14^{Ba}	1.46 ± 0.01^{Bb}	1.32 ± 0.02^{Bc}	1.18 ± 0.02^{Bd}	0.81 ± 0.01^{Be}	0.70 ± 0.01^{Bf}	0.49 ± 0.00^{Bg}
6	2.39 ± 0.03^{Ca}	1.14 ± 0.00^{Cb}	0.85 ± 0.01^{Cc}	0.67 ± 0.01^{Cd}	0.63 ± 0.01^{Ce}	0.49 ± 0.00^{Cf}	0.36 ± 0.00^{Cg}
9	1.24 ± 0.01^{Da}	0.78 ± 0.01^{Db}	0.23 ± 0.01^{Dc}	0.25 ± 0.02^{Dd}	0.27 ± 0.02^{De}	0.30 ± 0.01^{Df}	0.30 ± 0.02^{Dg}
12	0.08 ± 0.00^{Ea}	0.28 ± 0.00^{Eb}	0.16 ± 0.01^{Ec}	0.18 ± 0.01^{Ed}	0.19 ± 0.00^{Ee}	0.27 ± 0.00^{Ef}	0.27 ± 0.00^{Eg}

-Within each column: the different large letters of WC are significantly ($p<0.05$); within each row: the different small letters of WC are significantly ($P < 0.05$).

Table 2 shows the survival percentage of cryopreserved protocorms assessed by TTC staining as affected by preculture/pretreatment and dehydration time. It was noted that without preculture or dehydration or both, a very low percentage of the cryopreserved protocorms was observed. Meanwhile, pretreatment with 1.5 M sucrose with subsequent dehydration gave rise to higher percentages of viability of the cryopreserved protocorms. In most cases, the increase in the length of dehydration time resulted in an increment in the survival percentage of the cryopreserved protocorms. Increasing the level of sucrose concentrations in the preculture steps was also found to increase the survival percentage of the cryopreserved protocorms. However, a much too high level of sucrose concentration along with prolonged dehydration time were noted to cause negative effects on the viability of the cryopreserved protocorms.The highest survival percentage

of highest survival percentage of 86.67% was observed for encapsulated protocorms that were primarily subjected to pretreatment with 0.75 M sucrose followed by pretreatment with 1.5 M sucrose before dehydration for 12 h.

Table 2. Survival percentage of the cryopreserved *C. finlaysonianum* protocorms primarily subjected to sucrose preculture/pretreatment and dehydration.

Dehydration Time (h)	Survival (%) ±SE						
	Controls		Concentrations of Sucrose				
	1	2	0M	0.25M	0.5M	0.75M	1M
0	2.22 ± 1.11^{Bd}	11.11 ± 1.11^{Babc}	13.33 ± 0.00^{Bbc}	21.11 ± 1.11^{Bbc}	23.33 ± 0.00^{Bab}	36.67 ± 0.00^{Ba}	41.11 ± 1.11^{Bc}
3	6.67 ± 1.92^{Bd}	42.22 ± 1.11^{Babc}	30.00 ± 0.00^{Bbc}	36.67 ± 1.92^{Bbc}	40.00 ± 1.92^{Bab}	42.22 ± 1.11^{Ba}	27.78 ± 1.11^{Bc}
6	2.22 ± 1.11^{Bd}	53.33 ± 1.92^{Babc}	30.00 ± 0.00^{Bbc}	40.00 ± 0.09^{Bbc}	46.67 ± 1.92^{Bab}	48.89 ± 1.11^{Ba}	25.56 ± 1.11^{B}
9	3.33 ± 0.00^{ABd}	56.67 ± 0.00^{ABabc}	45.67 ± 1.00^{ABbc}	50.00 ± 0.00^{ABbc}	71.11 ± 1.11^{ABab}	45.56 ± 1.11^{ABa}	40.00 ± 1.92^{ABc}
12	0.00 ± 0.00^{Ad}	62.22 ± 1.11^{Aabc}	44.44 ± 1.11^{Abc}	70.00 ± 0.00^{Abc}	54.44 ± 1.11^{Aab}	86.67 ± 0.00^{Aa}	42.22 ± 1.11^{Ac}

-Within each column: the different large letters of survival are significantly (p<0.05); within each row: the different small letters of survival are significantly (P < 0.05).

Other than evaluating the survival percentage of cryopreserved protocorms based on TTC staining, determination of the recovery/regrowth percentage of cryopreserved protocorms on selected medium was also conducted and the results are given in Table 3. Immersion of encapsulated protocorms into liquid nitrogen without preculture/pretreatment and dehydration resulted in 0% recovery/regrowth of the cryopreserved protocorms. Even with preculture/pretreatment, dehydration of encapsulated protocorms for 3-6 h was insufficient for the recovery/regrowth of the cryopreserved protocorms. In some treatments, extension of the dehydration time to 9 h was noted to allow recovery/regrowth of the cryopreserved protocorms. As expected, dehydration of precultured or pretreated protocorms for 12 h gave rise to a marked recovery/regrowth of the cryopreserved protocorms, with the highest recovery/regrowth percentage of 65.56% observed for those primarily precultured with 0.75 M sucrose followed by pretreatment with 1.5 M sucrose prior to dehydration for 12 h, which was consistent with their survival percentage (86.67%) assessed by TTC staining.

Table 3. Recovery/regrowth percentage of the cryopreserved *C. finlaysonianum* protocorms primarily subjected to sucrose preculture/ pretreatment and dehydration.

Dehydration Time (h)	Recovery (%) ±SE						
	Controls		Concentrations of Sucrose				
	1	2	0M	0.25M	0.5M	0.75M	1M
0	0.00 ± 0.00^{Aa}	0.00 ± 0.00^{Ad}	0.00 ± 0.00^{Ab}	0.00 ± 0.00^{Ac}	0.00 ± 0.00^{Af}	0.00 ± 0.00^{Ag}	0.00 ± 0.00^{Ae}
3	0.00 ± 0.00^{Aa}	0.00 ± 0.00^{Ad}	0.00 ± 0.00^{Ab}	0.00 ± 0.00^{Ac}	0.00 ± 0.00^{Af}	0.00 ± 0.00^{Ag}	0.00 ± 0.00^{Ae}
6	0.00 ± 0.00^{Aa}	0.00 ± 0.00^{Ad}	0.00 ± 0.00^{Ab}	0.00 ± 0.00^{Ac}	0.00 ± 0.00^{Af}	0.00 ± 0.00^{Ag}	0.00 ± 0.00^{Ae}
9	0.00 ± 0.00^{Ba}	5.56 ± 1.11^{Bd}	3.33 ± 0.00^{Bb}	0.00 ± 0.00^{Bc}	4.44 ± 1.11^{Bf}	0.00 ± 0.00^{Bg}	0.00 ± 0.00^{Be}
12	0.00 ± 0.00^{Ca}	16.67 ± 0.00^{Cd}	21.11 ± 1.11^{Cb}	27.78 ± 1.11^{Cc}	57.78 ± 1.11^{Cf}	65.56 ± 1.11^{Cg}	42.22 ± 1.11^{Ce}

-Within each column: the different large letters of recovery are significantly (p<0.05); within each row: the different small letters of recovery are significantly (P < 0.05).

Figure 1 illustrates the morphological characteristics of the cryopreserved protocorms obtained after rapid thawing and orchid plants obtained following regrowth on selected medium. The cryopreserved protocorms obtained following rapid thawing were light green in color (Figure 1G). The cryopreserved protocorms were regrown on selected medium and development into dark green was observed after 6-8 weeks (Figures 1H and 1I) with plantlets achieved after 16 weeks (Figure 1J). Following 24-36 weeks of a regrowth process, the orchid plants raised from non-cryopreserved and cryopreserved protocorms exhibited no differences in their morphological characteristics (Figures 1K and 1L).

The overall results demonstrated that both the concentrations of sucrose in the preculture/pretreatment steps and the length of dehydration time had a profound effect on the water content of the encapsulated protocorms that determined their viability during the dehydration and freezing steps. Encapsulated protocorms with much too low and much high amounts of water were unlikely to survive under freezing conditions. In this study, the optimal water content for the two-month-old protocorms of *C. finlaysonianum* was 0.27 g H_2O/g DW.

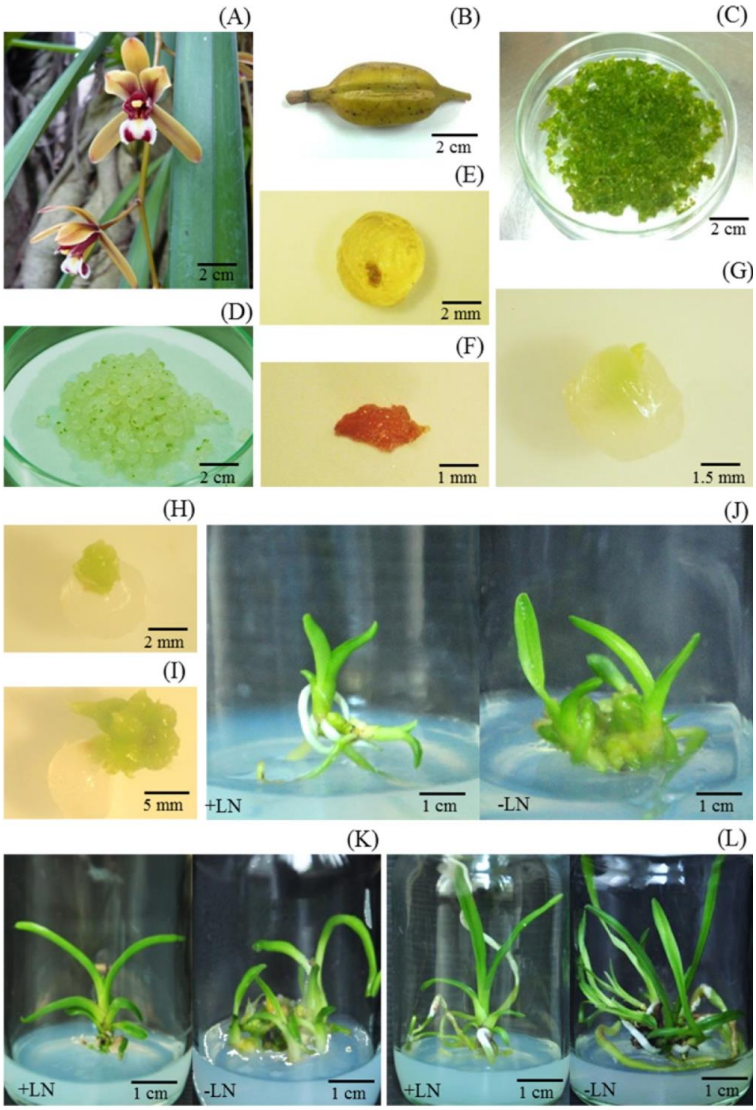

Figure 1. Cryopreservation protocorms of *C. finlaysonianum*: (A) Flower; (B) Fruit; (C) Protocorms for cryopreservation; (D) Encapsulate beads; (E-F) TTC staining of cryopre-served beads and protocorms; (G-I) Development of cry-opreserved protocorms after freezing for 1 week, 3 weeks, 8 weeks respectively; (J-L) Plantlets derived from cryo-preserved (+LN) and non-cryopreserved protocorms (-LN) for 16 weeks, 24 weeks and 36 weeks respectively. (Scale bar = 1mm-2cm)

3.2 Genetic stability of cryopreserved protocorms

Orchid plants raised from non-cryopreserved and cryopreserved protocorms were subjected to RAPD analysis using 45 random primers including OPA01-15, OPB01-15, and OPC01-15. Out of the 45 primers used, 43 primers revealed polymorphisms, producing a total of 229 fragments for the orchid plants raised from non-cryopreserved and cryopreserved protocorms. The other two primers (OPA10 and OPC04) failed to amplify DNA and therefore mono-morphic bands were not detected (Figures 2B and 2G). The size of DNA fragments ranged from 250 bp to >2000 bp while the number of amplified DNA bands varied form 0 to10 bands with an average of 5.36 bands per primer (Table 4). It was noted that the highest number of 10 bands was generated by the primer OPC08 (Figure 2H). More interestingly, only the PCR products amplified using the primer OPA14 exhibited polymorphic bands (Figure 2C). A 1900-bp DNA band was only detected in orchid plants raised from cryopreserved protocorms but was not found in those obtained from non-cryopreserved protocorms. Evaluation of the SI revealed a value of 0.998 between the orchid plants raised from non-cryopreserved and cryopreserved protocorms, which was very close to 1 indicating no genetic alterations in the cryopre-served protocorms.

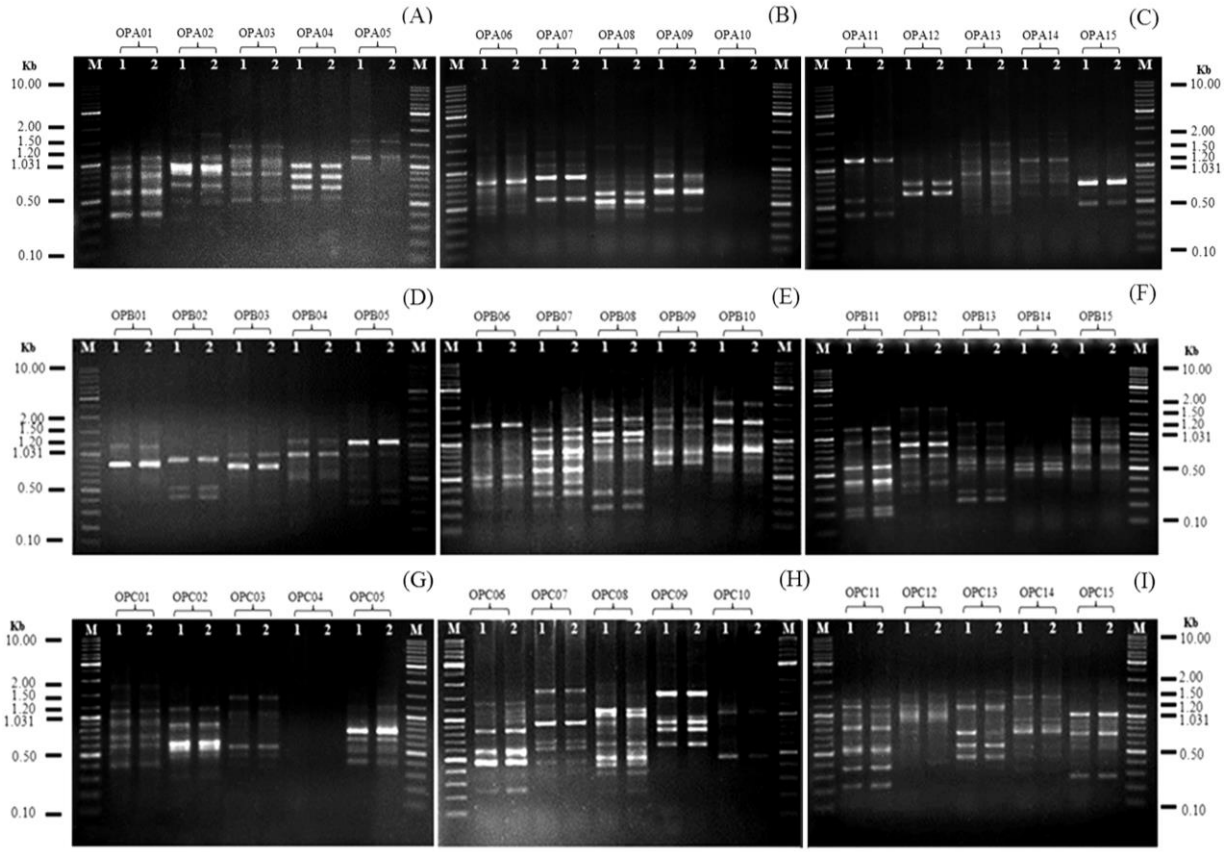

Figure 2. RAPD patterns of orchid plants raised from (1) non-cryopreserved and (2) cryopreserved protocorms of *C. finlaysonianum* using 45 primers: (A) primers OPA01-05; (B) primers OPA06-10; (C) primers OPA11-15; (D) primers OPB01-05; (E) primers OPB 06-10; (F) primers OPB11-15; (G) primers OPC01-05; (H) primers OPC06-10; (I) primers OPC11-15.

Table 4. RAPD primers used for RAPD genotyping.

Primers	Nucleotide sequence (5'to3'}	Range of amplicons (bp)	Total no. of bands	Polymorphic bands
OPA01	CAGGCCCTTC	350-1,100	7	-
OPA02	TGCCGAGCTG	500-1,700	7	-
OPA03	AGTCAGCCAC	500-1,400	5	-
OPA04	AATCGGGCTG	550-1,031	4	-
OPA05	AGGGGTCTTG	1,200-1,600	2	-
OPA06	GGTCCCTGAC	450-850	4	-
OPA07	GAAACGGGTG	600-1,400	6	-
OPA08	GTGACGTAGG	400-1,600	7	-
OPA09	GGGTAACGCC	450-900	3	-
OPA10	GTGATCGCAG	-	0	-
OPA11	CAATCGCCGT	450-1,200	4	-
OPA12	TCGGCGATAG	650-750	2	-
OPA13	CAGCACCCAC	400-1,500	7	-
OPA14	TCTGTGCTGG	600-1,900	6	1
OPA15	TTCCGAACCC	500-750	2	-
OPB01	GTTTCGCTCC	400-2,000	8	-
OPB02	TGATCCCTGG	600-1,500	3	-
OPB03	CATCCCCCTG	450-1,031	6	-
OPB04	GGACTGGAGT	350-1,200	7	-
OPB05	TGCGCCCTTC	500-1,400	6	-
OPB06	TGCTCTGCCC	850-1,200	2	-
OPB07	GGTGACGCAG	450-900	3	-
OPB08	GTCCACACGG	800-900	2	-
OPB09	TGGGGGACTC	550-1,100	3	-

Table 4. (Continued).

Primers	Nucleotide sequence (5'to3'}	Range of amplicons (bp)	Total no. of bands	Polymorphic bands
OPB10	CTGCTGGGAC	280-1,100	4	-
OPB11	GTAGACCCGT	450-1,400	6	-
OPB12	TTCCGACGCT	350-1,300	7	-
OPB13	TTCCCCCGCT	250-1,500	8	-
OPB14	TCCGCTCTGG	600-1,800	6	-
OPB15	GGAGGGTGTT	550-2,000	6	-
OPC01	TTCGAGCCAG	400-1,800	7	-
OPC02	GTGAGGCGTC	350-2,000	7	-
OPC03	GGGGGTCTTT	450-1,400	4	-
OPC04	CCGCATATAC	-	0	-
OPC05	GATGACCGCC	400-1,100	6	-
OPC06	GAACGGACTC	250-1,350	9	-
OPC07	GTCCCGACGA	450-1,700	7	-
OPC08	TGGACCGGTG	250-1,100	10	-
OPC09	CTCACCGTCC	600-1,500	5	-
OPC10	TGTCTGGGTG	470-1,050	2	-
OPC11	AAAGCTGCGG	250-1,400	8	-
OPC12	TGTCATCCCC	1031-1,100	2	-
OPC13	AAGCCTCGTC	450-1,500	5	-
OPC14	TGCGTGCTTG	450-1,450	7	-
OPC15	GACGGATCAG	280-1,150	7	-

4. Discussion

Any protocols proposed for the encapsulation-dehydration based cryopreservation of plant materials rely on optimization of sucrose concentrations in the preculture/pretreatment step as well as finding the optimal length of dehydration time in order to lower the water content in the plant materials to a level the materials are not damaged as a result of the freezing temperature. In general, the water content in plant materials determines their survival and regrowth ability under freezing conditions. High water content allows the formation of intracellular ice crystals within normal cells (Bain *et al.*, 2002). This results in increased permeability of the cell membrane that allows solute or electrolyte leakage and the subsequent interruption of ion stability in the plant cells which leads to chilling injury and cell death. Therefore, sucrose-based preculture/pretreatment and dehydration are pivotal to the successful cryopreservation of plant germplasms using encapsulation-dehydration technique.

In our study, protocorms of *C. finlaysonianum* were precultured with high sucrose concentrations, encapsulated into Ca-alginate, and pretreated with a high sucrose concentration again in order to lower the water content of the protocorms within the beads. A 4.1-fold decrease in the water content was achieved. The precultured/ pretreated encapsulated protocorms were then subjected to dehydration utilizing an air-drying method to further lower the water content. Our results revealed that dehydration alone could contribute to a profound reduction in the water content with a 61-fold decrease observed for a 12-h dehydration process (Table 1). However, our findings demonstrated that preculture of the two-month-old *C. finlaysonianum* protocorms with 0.75 M sucrose before encapsulation in Ca-alginate followed by pretreatment with 1.5 M sucrose and 12-h dehydration contributed to the highest level of viability as assessed by TTC staining. Furthermore, recovery/regrowth at 65.56% was achieved in the cryopreserved protocorms in which the water content was 0.27 g H_2O/g DW (21.15%). The findings obtained in our study were well-supported by one previous study (Engelmann, 2010) that reported high survival percentages were seen along with fast regrowth of cryopreserved explants in those with a water content of approximately 20%. Popova *et al.* (2016) also investigated the optimal water content within dehydrated beads and noted that a water content in a range of 20-43% contributed to a successful survival percentage of 11-67% with the highest level of viability of 67% observed for a water content of 20%. On the other hand, Kulus and Zalewska (2014) described that the level of water contents should be <20% and 20-40% fresh weight in pollen grains and seeds, respectively.

It is interesting to note that the sucrose concentration in the pretreatment step in our study was higher (1.5 M) than previous studies with a high viability of the protocorms. This indicated their tolerance to strong dehydration which was in good agreement with an earlier study (Hirano *et al.*, 2011) claiming high tolerance of the *C. finlaysonianum* seeds to dehydration. Hirano *et al.* (2006) also claimed that high concentrations of sucrose pretreatment with abscisic acid could enhance the survival of tissues and organs in the vitrification-based cryopreservation. Since sugrose acts as a potential osmotic dehydrating agent, it has been used in many cryopreservation protocols with different results reported (Bain *et al.*, 2002; Lurswijidjarusa & Thammasiri, 2004; Verleysen *et al.*, 2004).

It is also interesting to note that based on our results, the percent recovery/regrowth of the cryopreserved protocorms was much lower than their percent viability assessed by TTC staining. This was possibly due to the fact that sucrose-based preculture/pretreatment as well as dehydration was insufficient to protect all of the protocorms from the freezing

step. The resultant death of some of the protocorms indicated that TTC staining can not be used to assure the regrowth ability of the cryopreserved protocorms. Cell death might also have resulted from excessive dehydration that led to plasmolysis or osmotic shock in the explants (Gonzalez-Arnao *et al.,* 2010). The results obtained in our study were in good agreement with an earlier study (Hirano *et al.,* 2011) that reported a lower percentage of germination of cryopreserved *C. finlaysonianum* seeds compared to their viability determined by TTC staining.

Many different protocols have been proposed for the encapsulation-dehydration based cryopreservation of a variety of orchid species with variations in their efficiencies. For instance, strikingly different efficiencies in the percent regeneration of cryopreserved protocorms utilizing the encapsulation-dehydration method have been well-documented in members of the genus *Dendrobium*, including *D. cruentum* (27%) (Thammasiri, 2008), *D. gratiosissimum* (67%) (Bunnag *et al.,* 2009), *D. heterocarpum* (8.33%) (Pimda & Bunnag, 2010), *D. virgineum* (57%) (Maneerattanarungroj, 2009), and *D. cariniferum* (0%) (Pornchuti & Thammasiri, 2008). Meanwhile, the encapsulation-dehydration method was shown to yield low-to-moderate regeneration capacity of the cryopreserved protocorms of the species *Vanda coerulea* (40%) (Jitsopakul *et al.,* 2008), *Oncidium bifolium* (11.30%) (Flachsland *et al.,* 2006) and *Grammatophyllum speciosum* (24%) (Sopalun *et al.,* 2010). On the other hand, high recovery capacity of cryopreserved protocorms has been reported for the species *C. eburneum* (72%) and *C. hookerianum* (70%) using the encapsulation-dehydration method (Gogoi *et al.,* 2013). Protocols for the encapsulation-dehydration based cryopreservation of protocorm-like bodies (PLBs) have also been proposed for many orchid species and a high regeneration capacity (92%) of cryopreserved PLBs has been reported for the hybrid *Cymbidium* Twilight Moon 'Day Light' (Teixeira da Silva, 2013), with low-to-moderate regeneration capacity observed for *Cleisostoma arietinum* (49%) (Maneerattanarungroj *et al.,* 2007), *D. nobile* (50.2%) (Mohanty *et al.,* 2012), *O. hamana* (30%) (Miao *et al.,* 2005), and *Phalaenopsis bellina* (30%) (Khoddamzadeh *et al.,* 2011).

For *C. finlaysonianum*, only the vitrification-based cryopreservation has been proposed for the seed explants with an efficiency of 75.90% (Hirano *et al.,* 2011). The protocol for the encapsulation-dehydration based cryopreservation of the *C. finlaysonianum* protocorms was therefore proposed in this study and a regrowth capacity of 65.56% was achieved. Even though the aforementioned protocol had high efficiency allowing a maximum percentage of seed germination of 75.90%, at least 8 weeks were required for the cryopreserved seeds to recover. This was much longer than the time the cryopreserved protocorms required (only 4 weeks) for their recovery and regrowth as observed in the present study.

Assessment of the genetic stability of orchid plants raised from cryopreserved protocorms is another important step in cryopreservation. In this study, following regrowth of the cryopreserved protocorms to produce healthy plants, the obtained plants were evaluated for their genetic stability using RAPD genotyping. From the RAPD results, the orchid plants raised from non-cryopreserved and cryopreserved protocorms showed a SI value of 0.998, which was very close to 1. This indicated that the established protocol in this study was promising since genetic alterations were not detected and no polymorphisms were found in the 6-month-old orchid plants. The findings obtained in this study were well-supported by an earlier study (Turner *et al.,* 2001) which elucidated that cryopreservation of *Anigozanthos viridis* based on the encapsulation-dehydration method conferred marked viability of the cryopreserved explants with no genetic alterations detected. RAPD analysis is a reliable technique used globally due to its advantages. These advantages include the uncomplicated and rapid procedure for analysis, cost-effectiveness, very low amounts of genomic DNA needed, no hazardous materials, and the suitability for samples with no previous cognizance of sequencing data (Williams *et al.,* 1990).

5. Conclusions

This study has proposed a promising protocol for cryopreservation of the *C. finlaysonianum* protocorms using the encapsulation-dehydration method. The results demonstrated that preculture/pretreatment and dehydration had a profound effect on the survival percentage and regrowth ability of cryopreserved protocorms. Too much sucrose along with an extended dehydration time resulted in a sharp decrease in water content, which usually led to low percentages of viability of cryopreserved protocorms. Even though the lowest water content was observed for encapsulated protocorms that were dehydrated for 12 h without preculture and pretreatment, viability of cryopreserved protocorms was not detected which indicated the important role of sucrose as a cryoprotectant. In this study, two-old-month protocorms precultured with 0.75 M sucrose, encapsulated into Ca-alginate, and pretreated with 1.5 M sucrose before dehydration for 12 h showed the a low water content of 0.27 g H_2O/g DW which exhibited the highest percentages of viability and regrowth. This level of water content was sufficient to allow high levels of viability and regrowth of the two-month-old protocorms of *C. finlaysonianum*. Moreover, RAPD analysis revealed the genetic stability of the cryopreserved protocorms, indicating that the proposed protocol was effective and promising.

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