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Research Article
Development of Cryopreservation Procedure Using Vitrification Method of *Cymbidium aloifolium* (L.) Sw., Medicinal Orchid

[1]Attachai Trunjaruen, [2]Pitakpong Maneerattanarungroj and [1]Worasitikulya Taratima

[1]Salt-Tolerant Rice Research Group, Department of Biology, Faculty of Science, Khon Kaen University, Khon Kaen, Thailand
[2]Faculty of Veterinary Medicine, Khon Kaen University, Khon Kaen, Thailand

Abstract

Background and Objective: *Cymbidium aloifolium* is a multipurpose orchid that is used as an ornamental and medicinal plant. This study determined the most effective protocol for protocorm vitrification. **Materials and Methods:** Protocorms size 2-3 mm were precultured using liquid ND medium containing 0, 0.5 and 1.0 M sucrose for 24 hrs before incubated in loading solution for 20 min and moved to PVS2 then dehydrated for 0, 1, 2, 3, 4 and 5 hrs at 0°C. Treated protocorms were plunged into LN immediately after treatment with PVS2 and kept for 24 hrs. After thawing, cryopreserved protocorms were recovered in unloading solution for 20 min before culture on solid ND medium. Suitable conditions for protocorm cryopreservation by vitrification were identified to estimate genetic stability using the RAPD technique and Genomic Template Stability (GTS) calculation. **Results:** Results demonstrated the interaction between sucrose concentration and PVS2 duration with both factors affecting the survival of *C. aloifolium* protocorms after regrowth on ND medium for 12 weeks. The results reveal that treatment of preculture in 0.5 M sucrose and 2 hrs of PVS2 gave significantly the highest survival percentage (22%) and regeneration percentage (13%). Both survival and regeneration percentages decreased when sucrose concentration and PVS2 exposure period were increased. Results showed differences in RAPD banding patterns between cryopreserved and non-cryopreserved plantlets. GTS value at 79.02% indicated a genetic alteration in cryopreserved plantlets but the variation in morphology was not observed. **Conclusion:** These finding useful for further development of protocols of *C. aloifolium* and other *Cymbidium* orchids.

Key words: Orchid preservation, vitrification, RAPD technique, genetic stability, cryopreservation

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Corresponding Author: Worasitikulya Taratima, Department of Biology, Faculty of Science, Khon Kaen University, Khon Kaen, Thailand

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cymbidium aloifolium (L.) Sw. Fig.1a is an epiphytic orchid distributed in Southeast Asia, Nepal, India, Bangladesh, Sri Lanka, South China, Hong Kong and almost all the provinces of Thailand[1] as an ornamental plant. In some countries, *C. aloifolium* has been used as a traditional medicine to relieve inflammation[2] and the chloroform extract has been shown to have antibacterial activity[3]. Although *C. aloifolium* is not classified as an endangered species, overexploitation by human activities, e.g. deforestation has reduced abundance in its natural habitat. Moreover, orchid diversity and distribution are threatened by global warming, decreasing number of pollinators and environmental change[4]. To protect the plant from overexploitation and illegal trade, *C. aloifolium* has been registered in Appendix II of CITES and international trade is now strictly controlled[5]. Developments of plant cryopreservation techniques are important to maintain the orchid populations and conserve their germplasm.

Cryopreservation is a proficient technique for long-term storage of biological materials at the extreme temperature of liquid nitrogen (LN; 196°C) to arrest biochemical pathways and cell activities. Theoretically, this technique allows conservation for an infinite period without genetic alteration[6,7]. Successful cryopreservation of orchids has been performed using various techniques, including encapsulation-dehydration[8,9], encapsulation-vitrification[10], vitrification[11] and droplet-vitrification[12]. However, research regarding the cryopreservation of *Cymbidium* species is limited and a simple and reliable method is required to conserve *C. aloifolium* germplasm.

Vitrification is a cryopreservation method whereby intracellular fluids are transformed into a glass state without water crystallization by exposure of plant materials to mixtures of cryoprotectants[13]. The method has been performed with various orchid species[7,13]. Vitrification is the simplest method for cryopreservation and can be applied to all types of orchid tissue[7]. Hence, here, the vitrification method was applied using *C. aloifolium* protocorms to identify the most effective protocol for protocorm vitrification and investigate the effects of sucrose concentrations during the preculture step and periods of PVS2 exposure. The Random Amplified Polymorphic DNA (RAPD) technique was employed to determine the genetic stability of the recovered *C. aloifolium* plants after cryopreservation.

MATERIALS AND METHODS

Study area: This research project was performed from August, 2019 to July, 2020.

Plant materials: Ten months old capsules of *C. aloifolium* in Fig.1b were cleaned by excision of dead tissue, washing with distilled water and plunging into 70% ethanol. The capsules were then sterilized by 20% (v/v) sodium hypochlorite (Clorox) with 2-3 drops of Tween 20 for 20 min with shaking and rinsed three times with sterile distilled water. Sterilized capsules were dissected and seeds were obtained and cultured on New Dogashima (ND) medium supplemented with 1% (w/v) sucrose and 1% (w/v) potato extract with pH adjusted to 5.4[14]. The medium was solidified by the addition of 0.8% (w/v) agar before autoclaving at 121°C for 15 min. Cultures were incubated at 25±2°C with a 16/8 hrs light/dark cycle providing photosynthetic photon flux density (PPFD) 40 μmol m^{-2} sec^{-1}. After culture for 60 days, *C. aloifolium* protocorms were obtained and used as plant materials in the vitrification method in Fig. 1c.

Cryopreservation using vitrification method: Protocorms size 2-3 mm were used as explants. The protocorms were precultured with preculture solutions as liquid ND medium containing 0, 0.5 and 1.0 M sucrose for 24 hrs. Precultured protocorms were moved to sterile cryovials and incubated in loading solution (LS; liquid ND medium containing 2 M glycerol and 0.4 M sucrose) for 20 min at 25±2°C. Then, the LS was removed and the cryovials were filled with plant vitrification solution 2 (PVS2), liquid ND medium containing 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) dimethyl sulfoxide (DMSO) and 0.4 M sucrose. Protocorms were dehydrated using PVS2 for 0, 1, 2, 3, 4 and 5 h at 0°C. Cryovials containing treated protocorms were plunged into LN immediately after treatment with PVS2 and kept for 24 hrs.

After the freezing step, frozen cryovials were thawed in 40±2°C water for 2 min. PVS2 was replaced by unloading solution containing liquid ND medium and 1.2 M sucrose for 20 min at 25±2°C. Then, the unloading solution was removed and protocorms were cultured on a solid ND medium. Cultures were kept in dark condition for 1 week before cultured in a 16/8 hrs (light/dark) condition for 11 weeks. Survival and regeneration percentages were recorded. Immediately after the thawing and unloading steps, 2,3,5-triphenyl tetrazolium chloride (TTC) solution was used to detect living protocorms

and viability percentage was calculated. Protocorms were immersed in TTC solution and incubated at 24°C for 24 hrs. The *in vitro* grown protocorms were used as the positive control. Protocorms treated with preculture solution, LS, PVS2, thawing, unloading solution and regrowth without immersion in LN were used as non-cryopreserved controls.

Data collection and statistical analysis: Viability percentage was measured using TTC solution immediately after unloading solution and survival and regeneration percentages were evaluated at 12 weeks after regrowth. Each treatment consisted of five replicates, with twenty protocorms per replicate. Data were assigned to the analysis of variance to investigate the main effects of sucrose concentrations in preculture solution, periods of PVS2 exposure and their interaction on protocorm vitrification of *C. aloifolium*. To identify the optimal treatment for *C. aloifolium* protocorms vitrification, data were subjected to a Completely Randomized Design (CRD) and one-way ANOVA. The differences of means were separated by Duncan's Multiple Range Test ($p<0.05$).

DNA extraction and RAPD analysis: Total genomic DNA was isolated from leaves of nine months old plants raised from cryopreserved and non-cryopreserved protocorms. Leaves (0.2 g) were frozen with LN and ground to powder in a mortar. The CTAB method was applied with some modifications for DNA extraction[15]. Quantity and purity of the extracted DNA were determined by a DeNovix DS-11 Spectrophotometer with quality and RNA contamination tested by gel electrophoresis in 0.8% agarose at 100V in 0.5X TBE buffer followed by ethidium bromide staining.

For RAPD analysis, 40 arbitrary 10-mer primers were applied in a polymerase chain reaction (PCR). Each PCR reaction consisted of a 50 ng DNA template, 1 µM 10 bp primer and 5 µL 2X Taq Master Mix (Vivantis). To adjust the final concentration of magnesium chloride ($MgCl_2$) to 2 mM, 0.1 µL of 50 mM $MgCl_2$ was supplemented into the reaction. The total volume of PCR reaction was 10 µL. Amplification was carried out in PCR Turbo Cycler (Blue-Ray Biotech), consisting of initial denaturing at 94°C for 5 min, 35 cycles of denaturing at 94°C for 1 min, annealing at 45°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 7 min. PCR products were separated in 1.5% (w/v) agarose gel in 0.5X TBE buffer followed by staining with ethidium bromide and visualization under UV light.

Prominent RAPD fragments were counted and recorded as binary data (presence/absence). The size of the bands was measured with UVI-1D software (UVITEC Cambridge) and the VC DNA ladder mix (100-10,000 bp; Vivantis) was used as the molecular weight reference. Binary data were used to calculate genomic template stability (GTS) as the following equation[16]:

$$\text{Genomic template stability (GTS \%)} = \frac{1-a}{n} \times 100$$

where, a is the number of polymorphic or change bands in each sample and n = the number of total bands in the control.

RESULTS

Cryopreservation using vitrification method: Unloaded protocorms were infused with TTC solution to detect the presence of life. Protocorms of positive control treatments infused with TTC solution showed red staining in Fig. 1d. TTC assay results indicated no cryopreserved protocorms with positive TTC results in any treatments in Fig. 1e. Therefore, data of viability percentage were absent. Data of survival and regeneration percentages were submitted to one-way ANOVA to identify the most suitable conditions for *C. aloifolium*. Protocorms treated with cryoprotectants but not immersed in LN were used as non-cryopreserved control. All cryopreserved treatments gave no the protocorms with positive TTC assay results in Fig. 1f. Results of one-way ANOVA showed that protocorms precultured in 0.5 M sucrose and exposed to PVS2 for 2 hrs gave the highest survival (22%) and regeneration (13%) ($p<0.001$) in Fig. 1g, while, with no surviving protocorms in the same treatment of the non-cryopreserved condition in Fig. 1h shown. *In vitro* grown plantlets and transplanted plants showed better growth Fig. 1i-j. After regrowth for 12 weeks, survival and regeneration percentages were recorded and assigned to the analysis of variance. Results demonstrated the interaction between sucrose concentrations and periods of PVS2 exposure ($p<0.05$), while survival and regeneration percentages were affected by both factors ($p<0.001$). Survival and regeneration percentages of 0.5 M sucrose were higher than 0 and 1 M sucrose for all periods of PVS2 exposure in Fig. 2a-b. Significant differences in survival and regeneration percentages were observed for treatments of 0.5 M sucrose ($p<0.001$) with the highest recorded for treatment of 2 hrs PVS2 exposure but no significant

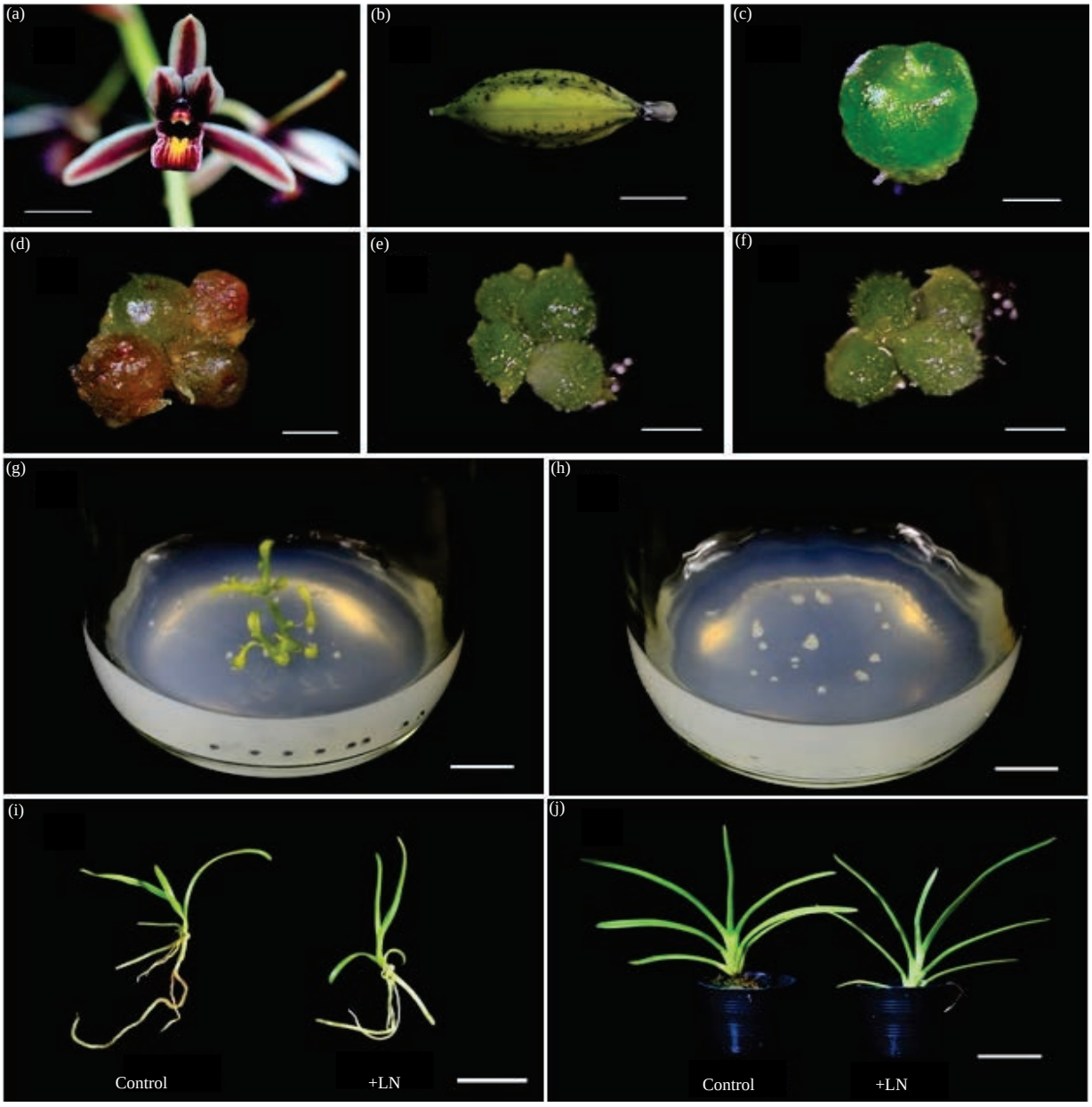

Fig. 1(a-j): (a) *Cymbidium aloifolium* flower (bar = 1 cm), (b) *C. aloifolium* capsule (bar = 2 cm), (c) 60 days old *C. aloifolium* protocorms (bar = 0.5 cm), (d) Positive control of TTC assay (bar = 1 mm), (e) TTC staining of cryopreserved protocorm (bar = 1 mm), (f) TTC staining of non-cryopreserved protocorm (bar = 1 mm), (g) Living protocorms from treatment of 0.5 M sucrose and 2 hrs PVS2 exposure of cryopreserved condition (bar = 1 cm), (h) Protocorms from treatment of 0.5 M sucrose and 2 hrs PVS2 exposure of non-cryopreserved condition (bar = 1 cm), (i) *In vitro* grown plantlets (+LN = cryopreserved plantlet; bar = 5 cm) and (j) Transplanted plants (+LN = cryopreserved plant; bar = 5 cm)

differences were found for treatments of 0 and 1 M sucrose. These results indicated that 0.5 M sucrose and 2 hrs PVS2 exposure were the most effective treatments in the preculture and PVS2 exposure steps. Both survival and regeneration percentages decreased as PVS2 exposure duration increased. Results were consistent with the analysis of variance, indicating that the most suitable sucrose concentration in preculture solution was 0.5 M with a PVS2 duration of 2 hrs. Positive TTC results were not observed in any non-cryopreserved treatment condition. Significantly highest survival percentage (25%) was obtained from protocorms precultured in 0 M sucrose and exposed to PVS2 for 0 hr, while

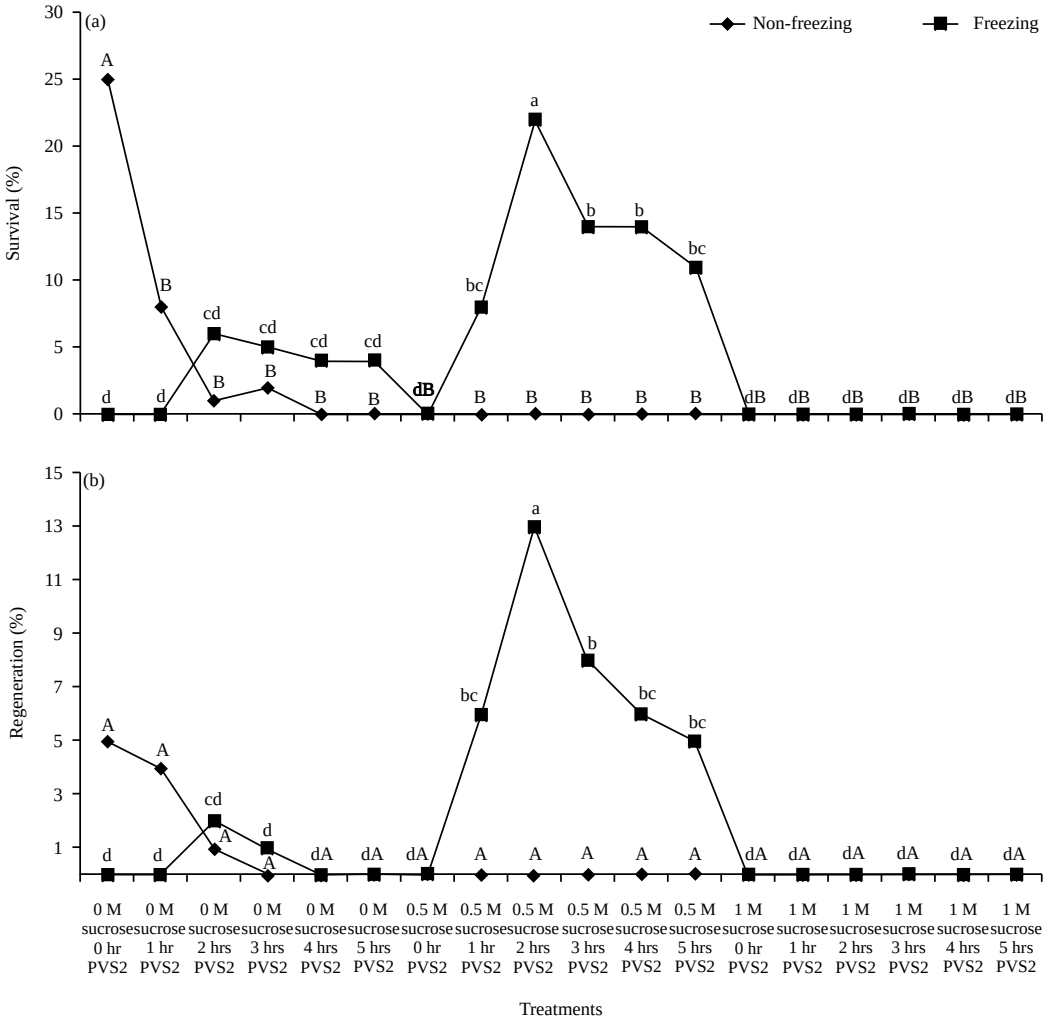

Fig. 2(a-b): (a) Survival and (b) Regeneration percentages of protocorms after culture for 12 weeks

Different lowercase and capital letters indicate significant difference at $p<0.05$ of cryopreserved and non-cryopreserved treatments, respectively. M: Molar, h: hour, PVS2: Plant vitrification solution 2

other treatments gave significantly lower percentages in Fig. 2a. Regeneration percentage was not significantly different among all treatments Fig. 2b. These results demonstrated that direct exposure of *C. aloifolium* protocorms to cryoprotectants during the dehydration and thawing steps resulted in lethal effects.

Genetic stability: Cryopreserved *C. aloifolium* protocorms were generated to plantlets and used in genetic stability estimation. Comparative RAPD banding profiles of non-cryopreserved (NC) and cryopreserved (C) plantlets using various primers were investigated and recorded in Fig. 3a-h. A total of 570 bands were produced using 40 random 10-mer primers, with 286 and 284 bands generated in non-cryopreserved and cryopreserved plantlets, respectively. The number of DNA fragments varied from 1 to 16 bands and the size of the amplified bands differed from 153-3,623 bp. Polymorphic bands were produced from 28 primers; OPA-02, OPA-03, OPA-05 in Fig. 3a; OPA-09, OPA-10 in Fig. 3b; OPB-08, OPB-09, OPB-10 in Fig. 3c; OPB-11, OPB-12, OPB-13, OPB-15 in Fig. 3d, OPC-04, OPC-05, OPC-06, OPC-07, OPC-08 in Fig. 3e; OPC-09, OPC-10, OPC-11, OPC-13 in Fig. 3f; OPD-03, OPD-05, OPD-07 in Fig. 3g; OPD-10, OPD-11, OPD-12, OPD-13 in Fig. 3h; while 12 primers generated monomorphic bands given in Table 1. Genetic stability was evaluated using the GTS equation at 79.02%, giving 20.98% genetic variation of cryopreserved *C. aloifolium* plantlets. The total number of bands from NC samples and C samples were observed as 286 and 284 respectively. Moreover, the number of polymorphic bands were 60 in total. However, morphological differences were not observed between *in vitro* grown and transplanted cryopreserved and non-cryopreserved plants.

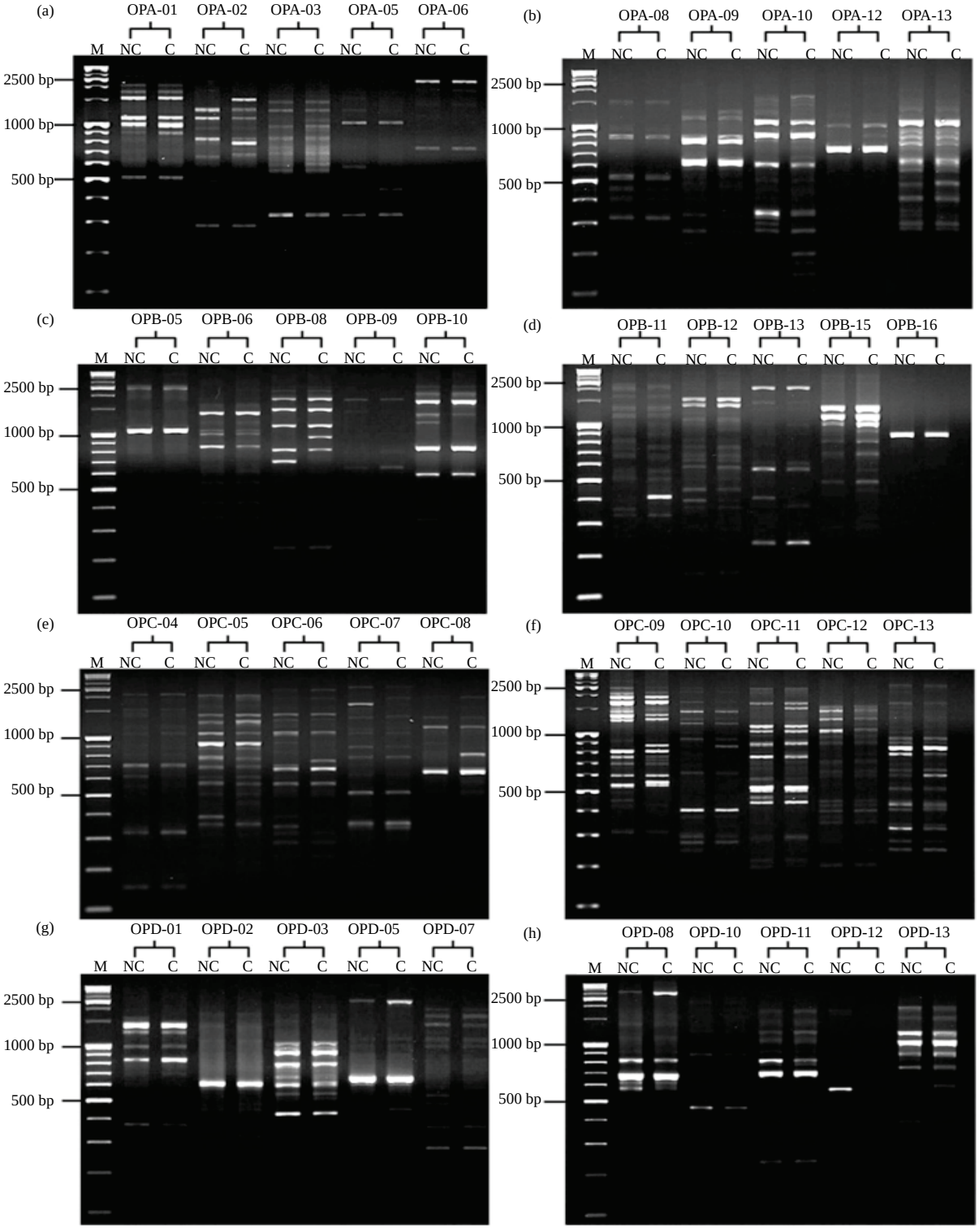

Fig. 3(a-h): RAPD banding profiles comparison of non-cryopreserved (NC) and cryopreserved (C) plantlets using various primers set

(a) OPA -01 to OPA-03, OPA-05, OPA-06, (b) OPA-08 to OPA-10, OPA-12, OPA-13, (c) OPB-05, OPB-06, OPB-08 to OPB-10, (d) OPB-11 to OPB-13, OPB-15, OPB-16, (e) OPC-04 to OPC-08, (f) OPC-09 to OPC-13, (g) OPD-01 to OPD-03, OPD-05, OPD-07 and (h) OPD-08, OPD-10 to OPD-13. M = DNA ladder

Table 1: RAPD profiles of non-cryopreserved (NC) and cryopreserved plantlets (C)

Primers	Sequence (5'-3')	Range of amplicon (bp)	Number of bands from NC samples	Number of bands from C samples	Number of polymorphic bands
OPA-01	CAGGCCCTTC	253-2,792	9	9	-
OPA-02	TGCCGAGCTG	282-1,489	7	9	2
OPA-03	AGTCAGCCAC	235-1,432	9	11	2
OPA-05	AGGGGTCTTG	325-1,235	4	3	3
OPA-06	GGTCCCTGAC	733-2,347	2	2	-
OPA-08	GTGACGTAGG	317-1,500	5	5	-
OPA-09	GGGTAACGCC	268-1,241	6	4	2
OPA-10	GTGATCGCAG	200-1,685	6	7	3
OPA-12	TCGGCGATAG	767-1,013	2	2	-
OPA-13	CAGCACCCAC	282-1,332	11	11	-
OPB-05	TGCGCCCTTC	1,073-2,500	2	2	-
OPB-06	TGCTCTGCCC	356-1,405	8	8	-
OPB-08	GTCCACACGG	240-2,321	7	7	2
OPB-09	TGGGGGACTC	647-1,845	4	3	1
OPB-10	CTGCTGGGAC	350-2,579	8	7	1
OPB-11	GTAGACCCGT	335-2,315	12	11	3
OPB-12	CCTTGACGCA	158-1,550	11	11	2
OPB-13	TTCCCCCGCT	240-2,043	7	7	2
OPB-15	GGAGGGTGTT	505-1,333	6	8	2
OPB-16	TTTGCCCGGA	889	1	1	-
OPC-04	CCGCATCTAC	153-2,199	7	6	1
OPC-05	GATGACCGCC	356-2,129	12	11	1
OPC-06	GAACGGACTC	349-2,500	9	7	4
OPC-07	GTCCCGACGA	343-2,731	8	9	3
OPC-08	TGGACCGGTG	648-2,000	3	5	2
OPC-09	CTCACCGTCC	305-2,433	14	16	4
OPC-10	TGTCTGGGTG	247-2,399	12	10	4
OPC-11	AAAGCTGCGG	197-2,366	13	11	4
OPC-12	TGTCATCCCC	203-1,515	12	12	-
OPC-13	AAGCCTCGTC	249-2,627	15	14	1
OPD-01	ACCGCGAAGG	375-1,403	5	5	-
OPD-02	GGACCCAACC	274-612	4	4	-
OPD-03	GTCGCCGTCA	428-1,095	7	6	1
OPD-05	TGAGCGGACA	450-2,464	2	3	1
OPD-07	TTGGCACGGG	276-1,956	9	8	3
OPD-08	GTGTGCCCCA	578-3,203	5	5	-
OPD-10	GGTCTACACC	465-883	4	3	1
OPD-11	AGCGCCATTG	340-1,771	8	10	2
OPD-12	CACCGTATCC	572-865	3	2	1
OPD-13	GGGGTGACGA	394-1,904	7	9	2
	Total		286	284	60

bp: Base pair, NC: Non-cryopreserved, C: Cryopreserved plantlets

DISCUSSION

C. aloifolium protocorms were immersed in a preculture solution containing three different sucrose concentrations and dehydrated using PVS2 for six different periods of exposure before storage in LN. Positive TTC assay results were not observed in any treatments, contrasting with the study by Worrachottiyanon and Bunnag[5] where TTC assay was carried out with cryopreserved *Cymbidium finlaysonianum* protocorms immediately after unloading. However, for cryopreservation of *Vanda* Kasem's Delight, positive TTC assay results were obtained after unloading and culture for three weeks[11], since some types of plant material require regrowth to repair damage from cryoinjuries[17]. Contrastingly, here, although the TTC assay was not successful, cryopreserved protocorms were recovered after culture for 12 weeks.

Survival and regeneration percentage data were analyzed using analysis of variance. Results indicated that 0.5 M sucrose gave the highest survival and regeneration percentages compared with other sucrose concentrations. Mubbarakh *et al.*[18] found that preculture on 0.25 M sucrose was the suitable concentration for vitrification of *Brassidium* Shooting Star Orchid protocorm-like bodies (PLBs), but no recovered PLBs were found in the treatment of 0 M sucrose.

The preculture step is an important factor in plant cryopreservation because it can improve stress tolerance by stimulation of proline accumulation[13]. In addition, 1 M sucrose gave no living protocorms because too high sucrose concentration caused negative effects on plant cryopreservation[18]. Two hours of PVS2 exposure was the most suitable period for *C. aloifolium* protocorm vitrification. DMSO is the most important component in PVS2 because it can migrate through the membrane rapidly and protect the cells by reducing the freezing temperature[13]. However, the plasma membrane can be injured by higher concentrations of DMSO[19]. Therefore, prolonged periods of PVS2 exposure can have detrimental effects on plant materials. Rajasegar *et al.*[20] found that exposure of *Ascocenda* Wangsa Gold PLBs to PVS2 for 30 min gave the highest regrowth percentage but this decreased at PVS2 durations longer than 30 min. The same phenomenon was found in our study. A decrease in survival and regeneration percentages was recorded for treatments with PVS2 duration longer than 2 hrs. Therefore, the period of dehydration is the vital factor that should be considered.

Results of analysis of variance demonstrated only the effects of each factor; therefore, one-way ANOVA was employed to identify the optimal condition for *C. aloifolium* protocorm vitrification. Results of the non-cryopreserved condition confirmed TTC assay finding that direct exposure of protocorms to cryoprotectants during dehydration and thawing steps caused lethal effects. Although no research was available regarding *C. aloifolium* cryopreservation, Pradhan *et al.*[21] studied the storage of *C. aloifolium* artificial seeds at 4°C. Results showed that plantlet regeneration decreased when storage exceeded 30 days. Cryopreservation is, therefore, an alternative method for long term storage. Worrachottiyanon and Bunnag[5] cryopreserved *C. finlaysonianum* protocorms using encapsulation-dehydration and recorded 65% regrowth. The encapsulation-dehydration method was employed for cryopreservation of *C. hookerianum* and *C. eburneum* and provided 70-72% regeneration[22]. These results suggested that encapsulation techniques applied in cryopreservation can protect plant materials from dehydration. In confirmation, Gogoi *et al.*[23] found that the regeneration percentage of *C. eburneum* protocorms cryopreserved by encapsulation-vitrification was higher than the vitrification method, while exposed *C. aloifolium* protocorms to cryoprotectants directly and recorded low survival and regeneration percentages.

Genetic stability of regenerated plants is an important factor because the materials are always exposed to conditions during cryopreservation and regeneration procedures that can induce genetic alteration[13]. Here, assessment of genetic stability was carried out using the RAPD technique and GTS value was determined at 79.02%, indicating variation of cryopreserved *C. aloifolium* plantlets compared with the non-cryopreserved control. Several studies indicated that cryopreservation procedures did not affect plant genetic stability such as blueberry[24] and *C. finlaysonianum*[5]. Current results differed from these and supported earlier studies cryopreservation of *Chrysanthemum x morifolium*, aberration of DNA banding patterns was observed in the preculture step for 0.3 M sucrose detected by RAPD technique[25]. Results from these studies suggest that cryoprotectants used in the vitrification method can induce genetic alteration. Current results confirmed that toxicity of the cryoprotectants affected survival and regeneration percentages of cryopreserved *C. aloifolium* protocorms and also the genetic stability of cryopreserved plantlets; however, genetic alteration did not induce abnormality in plant morphology after culture and transplantation.

CONCLUSION

This study exhibits an alternative cryopreservation process of *C. aloifolium* protocorms by using the vitrification system. Results of analysis of variance confirmed that preculture in sucrose and dehydration using PVS2 were critical vitrification steps. One-way ANOVA determined the combination of 0.5 M sucrose concentration in the preculture step and 2 hrs PVS2 exposures as the most effective protocol for vitrification of *C. aloifolium* protocorms. Results of the non-cryopreserved condition confirmed that direct exposure to cryopreservation can severely damage plant materials. RAPD analysis revealed genetic alteration in cryopreserved *C. aloifolium* plantlets. Protocorms were directly immersed in PVS2 containing DMSO which induced mutagenesis. Current results are useful to develop a more effective protocol for the cryopreservation system of *C. aloifolium* which can be modified and applied to conserve other *Cymbidium* species.

SIGNIFICANCE STATEMENT

This study discovered a suitable protocol that can be beneficial for *C. aloifolium* cryopreservation. This study will help the researchers to uncover the critical areas of the vitrification system of *C. aloifolium* protocorms that many researchers were not able to explore. Thus, a new theory on *C. aloifolium* protocorms cryopreservation using the vitrification technique may be arrived at.

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