Asia-Pacific Journal of Science and Technology: Volume: 25. Issue: 03. Article ID.: APST-25-03-05. Research Article

APST

Asia-Pacific Journal of Science and Technology

https://www.tci-thaijo.org/index.php/APST/index

Published by the Research and Graduate Studies,
Khon Kaen University, Thailand

The effects of vitrification method on the seed survival and genetic stability of *Rhynchostylis gigantea* (Lindl.) Ridl.

Duangrath Jariyajirawattana[1] and Sumontip Bunnag[2,*]

[1]Department of Biology, Faculty of Science, Khon Kaen University, Khon Kaen, Thailand
[2]Applied Taxonomic Research Center (ATRC), Department of Biology, Faculty of Science, Khon Kaen University, Khon Kaen, Thailand
[*]Corresponding author: sumbun@kku.ac.th

Received 10 July 2019
Revised 20 May 2020
Accepted 20 May 2020

Abstract

Orchids are highly important and valuable plants due to their diversity and the beauty of their flowers. Presently, there are many orchid species, which are going to become extinct due to environmental changes and over-collection for commercial uses. Cryopreservation is an alternative method for the storage of plant genetic resources. The aim of this study was to evaluate the effectiveness of the vitrification method for the cryopreservation of *Rhynchostylis gigantea* seeds. The seeds were pre-cultured for 24 h in a liquid New Dogashima (ND) medium, which had been supplemented with 0.5 M sucrose; incubated with a mixture of 0.4 M sucrose and 2 M glycerol for 30 min; and then finally dehydrated with plant verification solution 2 (PVS2) for 0-120 min at 25 ˚C prior to storage in liquid nitrogen (LN) for 24 h. The orchid seeds were successfully preserved by the vitrification method. The results found that the optimal dehydration time in PVS2 had been 30 min, which gave the highest recovery rate of 61% after culturing the seeds on ND medium for 30 d. The results of genetic stability assessment revealed that when comparing the cryopreserved and non-cryopreserved seeds, the Random amplified polymorphic DNA (RAPD) patterns had been similar. A total of 92.5% of the RAPD primers had given a high SI value, which ranged between 0.9 and 1.0 and indicated that the genetic stability of seeds stored in liquid nitrogen had been well-maintained.

Keywords: Cryopreservation, Genetic stability, Seeds, *Rhynchostylis gigantea*

1. Introduction

Orchidaceae is the largest family of flowering plants, has more than 800 genera and approximately 17,000-35,000 species [1,2]. Orchids are well-known and regularly used in different countries of the world. They have the highest value among cut-flower crops, which makes them important to Thai agriculture and economy [3]. *Rhynchostylis gigantea* is one of the orchid species, which is now at risk of becoming extinct due to their complex reproductive processes and the impact of environmental factors, such as global warming, flood, drought and stress, as well as the constant destruction of orchid habitats leading to decreases in their genetic variability in nature [4]. Hence, *R. gigantea* is currently classified in the Appendix-II of the Convention in International Trade in Endangered Species of Wild Fauna and Flora (CITES).

Due to the large number of orchid species that are being threatened with extinction, a preservation method is necessary to ensure their conservation without losing their genetic variation. Cryopreservation (or *ex situ* conservation) is one of the major techniques for the preservation of endangered species because it requires the use of ultra-low temperatures (liquid nitrogen at -196 ˚C) [5]. At this temperature, all metabolic processes in the cell become inactive, which, therefore, allows for long periods of storage without any genetic variations and provides a safe and cost-effective method [6].

In recent years, cryopreservation techniques have been successfully developed for cryopreservation of orchid tissues/organs, such as immature seeds [5], seeds [7], protocorms [8], and protocorm-like bodies [9]. Among the cryopreservation techniques, vitrification has been widely developed for the following reasons: 1) it is easy to

use, 2) there is high reproducibility, and 3) it is an effective technique that can be applied to a wide range of plant species [10].

The first report of the use of vitrification method as the major cryopreservation technique with plant tissues was carried out by Uragami et al. (1989) [11]. The technique involved removing most of the water in the plant tissues, which could freeze, by exposing them to highly concentrated cryoprotective substances and performing dehydration with a plant vitrification solution (PVS), such as PVS2 [12]. The main problem encountered with this technique was the toxicity of the PVS2, which was due to the chemical components of the solution [6]. Hence, the optimal conditions for the cryopreserved seeds of *R. gigantea* has been investigated. Therefore, the objectives of this study were to evaluate the effectiveness of the vitrification method for cryopreservation of *R. gigantea* seeds and to investigate the genetic stability of the recovered plantlets, which had been derived from the cryopreserved seeds using the Random amplified polymorphic DNA (RAPD) technique.

2. Materials and methods

2.1 Plant materials

Mature capsules of *R. gigantea*, collected after 210 d of natural pollination, were derived from the Department of Biology at the Faculty of Science, Khon Kaen University in the Nai-Muang Sub-district of the Muang District in Khon Kaen Province, Thailand. The capsules were cleaned by washing them with detergent and then rinsing them with running tap water. Subsequently, the pods were dipped in 70% ethyl alcohol and then shaken in 25% sodium hypochlorite (NaOCl) for 30 min. After washing three times with sterile distilled water, the capsules were soaked in 95% ethyl alcohol and flamed. The sterilized capsules were cut longitudinally on a sterile Petri dish, and only the sterilized seeds were used as materials for this study.

Prior to the experiments, the characteristics of the capsules and seeds, such as capsule size, capsule weight, seed size, seed weight, and seed moisture content were all measured. The moisture content of the seeds was determined using a hot air oven at 103 ˚C for 17 h [13] and was then expressed in percentages per fresh weight.

2.2 Vitrification procedure and treatments

The sterilized seeds were pre-cultured in a liquid modified New Dogashima (ND) medium [14], which had been supplemented with 0.5 M sucrose and was left on a shaker (110 rpm) for 24 h at room temperature (25±2 ˚C). After removing the pre-culture solution, the seeds were then transferred into 1.8 ml cryotubes containing LS [15], which had been supplemented with 0.4 M sucrose and 2 M glycerol, and were then left for 30 min. To evaluate the optimum dehydration time in PVS2, LS was drained and the seeds were dehydrated at 25±2 ˚C for different exposure times (0, 30, 60, 90, and 120 min) with PVS2, which contained 0.4 M sucrose, 15% (v/v) ethylene glycol, 15% (v/v) dimethylsulfoxide (DMSO), and 30% (v/v) glycerol in ND medium (pH 5.4). The seeds were then directly plugged into liquid nitrogen (LN). Treatment control consisted of seeds cultured on a modified solid ND medium with no cryoprotective solution and directly immersed in LN. After 24 h in LN, the cryopreserved seeds were immediately re-warmed at 40±2 ˚C in water bath for 2 min. PVS2 was replaced by adding an unloading solution [16] containing ND medium with 1.2 M sucrose (pH 5.4), and the samples were then further left for 20 min. Treatments were comprised of six replicates with approximately 900-1000 seeds in each.

2.3 Viability assessment

The viability of the cryopreserved seeds was evaluated by the 2,3,5-triphenyltetrazolium chloride (TTC) staining test according to the protocol by Ishikawa et al. (1997) [17]. The seeds were rinsed three times with autoclaved distilled water and then incubated in a 1% (w/v) TTC solution for 48 h at 25±2 ˚C in the dark. The total number of red or pink seeds was counted as viable using a Nikon smz-2t stereomicroscope (Nikon Corporation, Japan). The experiment was repeated six times. The survival percentage was determined according to the formula below:

$$\text{Survival percentage (\%)} = \frac{\text{the number of red or pink seeds}}{\text{the total number of seeds}} \times 100 \quad (1)$$

2.4 Germination rate

For seeds germination, the seeds were cultured on a solidified ND medium, which had been supplemented with 10 g/L potato powder, 10 g/L sucrose, and 10 g/L agar, and which had a pH of 5.4. The cultured seeds were maintained in a culture room at 25±2 ˚C under a photoperiod of 16 h light/ 8 h dark with a photon dose of 40 µmol/m^2/s. The germination rates were determined by investigating the ability to re-grow the cryopreserved

seeds after 30 d of cryopreservation. The seeds were assessed to be viable if they showed obvious signs of greening and expansion.

2.5 Statistical analysis

The survival rate and germination rate of seeds after cryopreservation were analyzed using an analysis of variance (ANOVA). In order to compare the means of the treatments, the least significant difference (LSD) values were calculated at p=0.05.

2.6 Analysis of genetic stability

2.6.1 DNA extraction and quantification

The six-month-old plantlet leaves of the clone plants, which had been derived from control and cryopreserved seeds, were harvested and stored in zip-lock plastic bags at -80 ˚C until DNA extraction. Approximately 100 mg of the leaf sample was ground into a fine powder in LN. The DNA extraction kit, GenElute™ Plant Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis, USA), was used to extract the total genomic DNA, according to the manufacturer's recommendations. The quality of the extracted DNA was assessed on 1.0% (w/v) agarose gel and stained with ethidium bromide (EtBr), while the quantity of that was also compared with known amount of lambda DNA markers (50, 70, 100, and 140 ng/µL). The DNA samples were diluted to 50 ng/µL in TE buffer and then stored at 4 ˚C for used as DNA template in PCR amplification.

2.6.2 RAPD amplification

The genetic stability assessment of both groups from cloned plants was performed using random RAPD primers (Operon Technologies Inc., USA). The forty RAPD primers, which were used in PCR amplification, are listed in Table 3. The PCR reaction was carried out in a sterile 0.2 ml tube (10 µl) containing 50 ng of genomic DNA, 0.8 µM of each RAPD primer, 1x of GoTaq® Green Master Mix (Promega, USA), 5 mM $MgCl_2$, and 7.5 µl of sterile deionized water to adjust the volume of the PCR reaction. PCR amplification reactions were conducted on the PCR Express Thermal Cycler (Thermo Hybaid, USA). The amplification condition was used as follows: initial denaturation 94 ˚C for 3 min, followed by 35 cycles of 94 ˚C for 1 min, 38 ˚C for 1 min, 72 ˚C for 2 min, and the final extension step was at 72 ˚C for 10 min. To confirm the absence of contamination in the PCR reaction, negative controls were conducted by adding all components with the exception of the DNA template. The PCR products were then stored at 4 ˚C prior to gel electrophoresis.

2.6.3 DNA electrophoresis

About 8 µl of amplified PCR products was separated on 1.5% (w/v) agarose gel in 1×TBE buffer (Vivantis, Malaysia). The electrophoresis was carried out at 100 V for 1.30 h using MUPID-exU Horizontal electrophoresis System (Mupid, Japan). Agarose gels were subsequently soaked in 0.025 µg/mL EtBr solution for 10 min and were then rinsed in deionized water for 30 sec. The gels were visualized and photographed with the Gel documentation transilluminator (UVITEC Gel Documentation Systems, UK).

2.6.4 Data analysis

The presence of amplified DNA bands was analyzed with the UVI-1D software (UVITEC, UK). DNA fragments, which were well-resolved and unambiguous, were marked and computed using the sizes against the standard DNA marker, VC DNA ladder mix (Vivantis, Malaysia). The DNA patterns of each treatment group were evaluated by calculating the Similarity Index (SI) of the cryopreserved seeds as compared to the non-cryopreserved seeds. The reproducible bands were scored based on the presence (1) or absence (0) of the bands. The coefficients of similarity between those groups were calculated using the formula below [18]:

$$Similarity\ index\ (SI) = \frac{2N_{xy}}{N_x + N_y} \quad (2)$$

N_{xy} The number of monomorphic bands between the cryopreserved and non-cryopreserved seeds
N_x The total number of bands in the non-cryopreserved seeds
N_y The total number of bands in the cryopreserved seeds

3. Results and discussion

3.1 Cryopreservation of R. gigantea seeds

The characteristics of the *R. gigantea* seeds harvested after 210 d of natural pollination are presented in Table 1. The mature capsules were yellowish-green in color with a size of 4.38×1.2 mm in length. The moisture content of the tiny-brown seeds was 18.53% of the fresh weight prior to cryopreservation. To confirm the survival of the fresh seeds, TTC staining test was carried out (81.5% viability) and the seeds were then directly cultured onto solidified ND medium, yielding a germination rate of 82.1% (Table 2). It was noted that the moisture content of the fresh seeds in this study was likely to be low when compared with other orchid species, e.g., *V. tricolor* [19] and *V. coerulea* [20]. However, our results were similar to findings from Galdiano et al. (2013) [21], who revealed that the moisture content of the seeds of *Oncidium flexuosum* after harvesting had been 11.3%, which yielded a seed germination of 91%. Consequently, having a low water content in the natural seeds may facilitate successful cryopreservation [22].

Table 1 The characteristics of *R. gigantea* used in the experiments.

Characteristics	
Capsule size[a] (*L* × *W*, cm)	4.38 ×1.2
Capsule weight[a] (g)	1.83
Seed size[b], (*L* × *W*, mm)	0.23 × 0.08
Seed weight of 100 seeds[c] (mg)	1.02
Seed moisture content[c] (% fresh weight)	18.53

L: length; W: width; [a]average from 4 capsules; [b]average from 100 seeds; [c]average from 6 replications.

Table 2 The effects of the dehydration time in PVS2 upon the viability rates, germination rates, and germination times of *R. gigantea* after cryopreservation.

Seeds	Control	Dehydration time in PVS2 (min)				
		0	30	60	90	120
Viability (%)	81.5±0.7[a]	0.0±0.0[f]	69.4±0.2[a,b]	49.9±7.6[c,d]	25.39±2.5[e]	2.2±0.4[f]
Germination (%)	82.1±5.8[a]	0.0±0.0[f]	61.3±4.7[b,c]	32.3±3.0[e]	35.33±2.2[d,e]	4.7±1.5[f]
Germination time (d)	67	0	24	24	24	24

Data represents the means from six replications. Means with the same letters are not significantly different using Tukey's test at $p<0.05$.

The germination of cryopreserved seeds of *R. gigantea* was more rapid than the non-cryopreserved seeds. After the seeds had been sown on solidified ND medium, the greening and expansion of the cryopreserved seeds occurred after 24 d, which was more rapid than the non-cryopreserved seeds (67 d). The obtained results were supported by findings from a study by Jitsopakul *et al.* (2012) [19], who reported that the germination of *V. tricolor* seeds subjected to vitrification (28 d) had been faster than the non-cryopreserved seeds (60 d). This could be due to the fact that freezing might activate a decrease in enzymatic reactions and metabolic processes through a dormancy mechanism [23].

After treating seeds with the unloading solution, the seeds stored in LN were rinsed with sterile distilled water prior to conducting a viability assessment with the TTC staining test. The results of the staining of the non-cryopreserved and cryopreserved seeds with TTC solution at 25 ˚C for 48 h in the dark are shown in Figures 1D and 1E, respectively. It was noted that the rose-red seeds had only been obtained from the cryopreserved seeds, while none had been observed in non-cryopreserved seeds. This result revealed that the non-cryopreserved seeds had been damaged by the formation of ice crystals in their cells when immersed in the LN. In orchid seeds, the TTC staining test has been previously used to estimate seed viability [5,24-25]. In our study, the optimal dehydration time in PVS2 was 30 min, which had given the highest TTC staining at 69.4% and was higher than the germination test (Table 2). This was due to the fact that the damage that had occurred to the seeds during storage in LN might have promoted the production of the dehydrogenase enzyme [24]. Therefore, the TTC test was not found to be a precise predictor of germination for *R. gigantea* seeds after vitrification. This result was supported by findings from a study by Hu et al. (2013) [25], who noted that seed viabilities of *B. formosana*, which were evaluated by the germination test, had been lower than the TTC test. From the obtained results, it was concluded that the accuracy of the survival rate obtained from the TTC staining method actually depends upon the orchid species. Hence, to confirm the accuracy of seed survival after the vitrification method, the cryopreserved seeds should be examined simultaneously by using two different viability tests: the TTC method and the germination test [26].

The effects of the dehydration time in PVS2 on the germination rates of the cryopreserved seeds by the vitrification method are shown in Table 2. No germination was observed in seeds without treatment with cryoprotective solution before being plugged into LN. As for the cryopreserved seeds, the highest germination

was achieved at 61.3% . This occurred after the seeds had been pre-cultured in medium with 0.5 M sucrose, which was followed by pretreatment with a mixture of 0.4 M glucose and 2 M glycerol, and then was finally dehydrated for 30 min in PVS2. In the vitrification method, increments of the survival of the cryopreserved seeds could be induced by preculture with high levels of osmotic substances (e. g. , sucrose, sorbitol, and mannitol) [24]. This was due to the fact that preculture promotes the accumulation of sugar in the cell, which results in increases in the stability of the membranes under dehydration conditions [27]. Moreover, it has been revealed that LS containing 0.4 M glucose and 2 M glycerol has been successfully used with various orchid species because the solution can protect the membrane during freezing [17,24]. In regard to the dehydration time of PVS2, the germination rate of the seeds had declined when the dehydration time was raised. When the incubation time was extended to 120 min, it was 4.7%. The results suggested that the excessive exposure time to PVS2 had resulted in the accumulation of chemical toxicity, such as DMSO in the cells [19,24]. Hence, the optimal exposure time to PVS2 is important for seed survival after cryopreservation by vitrification. The application of the vitrification method to successfully cryopreserve seeds of orchid species has been reported in *B. striata* [5], *Dendrobium* hybrids [13], *V. tricolor* [19], *Vanda coerulea* [20], *O. flexuosum* [21], and in *B. formosana* [25].

Figures 1F-1K represent the morphological characteristics of the cryopreserved seeds following treatment with vitrification and storage in LN. The cryopreserved seeds were then re-cultured on solidified ND medium in the culture room at 25 ˚C and the seeds, which turned into a small yellowish-green color, were observed after 8 weeks (Figure 1F). After two subcultures into fresh ND medium, the germinating seeds were able to develop into normal seedlings with the green leaves (Figure 1G-1H). At 28 weeks after re-growing, the plantlets developed from non-cryopreserved and cryopreserved seeds were found to be healthy. Moreover, no morphological abnormalities were observed (Figure 1I-1K).

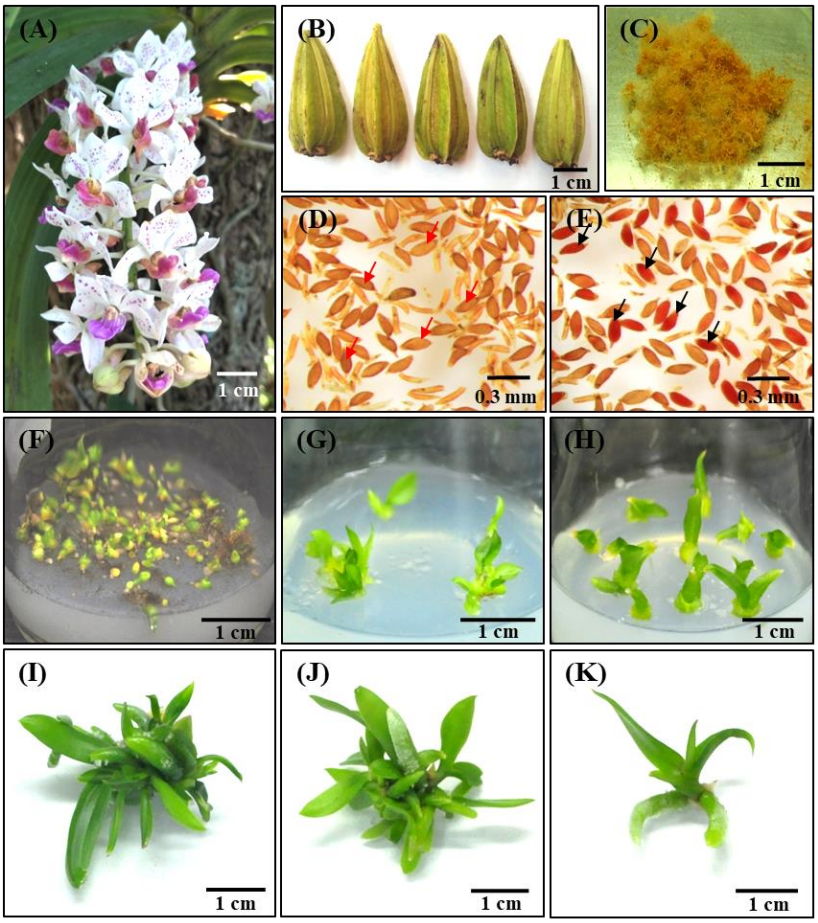

Figure 1 The characteristics and developmental stages of *R. gigantea* seeds subjected to the vitrification method. (A): flowers, (B): mature capsules, (C): non-germinated seeds, (D-E): TTC staining of non-cryopreserved (-LN, left) and cryopreserved seeds (+LN, right) (*black arrows* indicate viable seeds; *red arrows* indicate non-viable seeds), (F): 8 week-old plantlets on solid ND medium, (G-H): plantlets developed from non-cryopreserved and cryopreserved seeds at 20 weeks, respectively, (I): non-cryopreserved plantlets, and (J-K): cryopreserved plantlets after recovery for 28 weeks.

3.2 Genetic stability

A number of molecular techniques have been used to evaluate the genetic stability of cryopreserved plants. RAPD is one reliable technique given that it is easy to use, cheap, cost-effective, and requires only a small amount of DNA template. In addition, it is suitable for the samples with no previous DNA sequence data [28]. In the present study, the 6-month-old plantlets, which were healthy and had no abnormalities, were used as a material for PCR amplification in order to assess the RAPD profiles. The results from both the cryopreserved and non-cryopreserved seeds of *R. gigantea* revealed that a total of 40 primers could be the amplified genomic DNA and that they could produce sharp and clear reproducible fragments as shown in Figure 2.

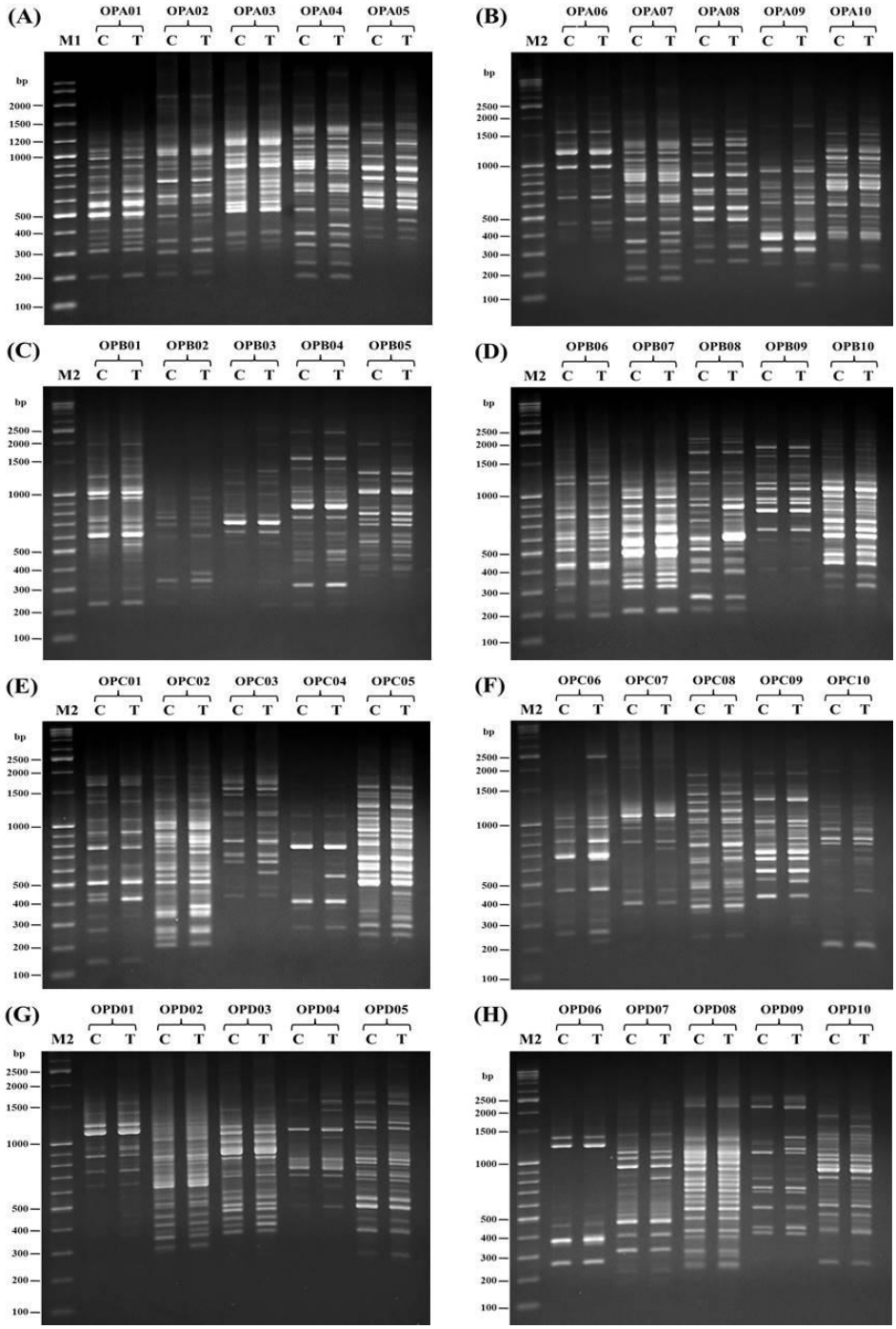

Figure 2 The RAPD results of *in vitro* plantlets derived from the control (C) and the cryopreserved (T) seeds of *R. gigantea* by 40 random primers; (A): OPA01-10, (B): OPA11-20, (C): OPB01-10, (D): OPB11-20, (E): OPC01-10, (F): OPC11-20, (G): OPD01-10, and (H): OPD11-20. Lane M1: VC 100 bp plus DNA ladder (Vivantis), lane M2: VC DNA ladder mix (Vivantis).

The total number of amplified DNA fragments obtained from the control plants had been 408 bands, while the number of bands from the cryopreserved plants had been 431, ranging from 4 bands (OPC-04) to 17 bands (OPC-05), with an average of 11.05 bands per primer. Out of the 442 bands from both groups, 397 bands (89.82 %) had been monomorphic and 45 bands (10.18 %) had been polymorphic with size range between 150 bp and 2500 bp. According to our calculations, most of the RAPD primers (92.5%) had given a high SI value, which varied between 0.9 and 1.0 (Table 3). The results demonstrated that no DNA alteration had been detected after cryopreservation, which indicated both the control and cryopreserved groups had demonstrated genetic stability. Our findings were also supported by the previous studies, such as a study conducted on *Dendrobium* hybrids, which had been derived from PLBs cryopreservation by vitrification [9,18]. Reviews indicated that the DNA patterns, which had been derived from cryopreserved explants by using the vitrification method, had been stable as compared to the non-cryopreserved explants. Therefore, the vitrification method is suitable for the preservation of *R. gigantea* seeds.

Table 3 The similarity indices from the RAPD analyses of DNA samples derived from seeds of *R. gigantea* subjected to the vitrification method.

Primers	Primer sequences (5´ - 3´)	Total no. of bands		Monomorphic bands	Polymorphic bands	Length of amplified DNA (bp)	Similarity index (SI)
		control plants	cryopreserved plants				
Kit OPA							
OPA-01	CAGGCCCTTC	12	12	12	0	210-1100	1.0
OPA-02	TGCCGAGCTG	13	12	12	1	220-2300	1.0
OPA-03	AGTCAGCCAC	14	13	13	1	320-1200	1.0
OPA-04	AATCGGGCTG	15	16	15	1	200-1300	1.0
OPA-05	AGGGGTCTTG	10	10	10	0	370-1200	1.0
OPA-06	GGTCCCTGAC	6	6	5	2	470-1700	0.8
OPA-07	GAAACGGGTG	15	16	15	1	180-1200	1.0
OPA-08	GTGACGTAGG	12	13	12	1	260-1700	1.0
OPA-09	GGGTAACGCC	10	11	9	3	150-1800	0.9
OPA-10	GTGATCGCAG	14	15	13	3	240-1600	0.9
Kit OPB							
OPB-01	GTTTCGCTCC	8	9	8	1	240-2000	0.9
OPB-02	TGATCCCTGG	5	8	5	3	340-1000	0.8
OPB-03	CATCCCCCTG	6	7	6	1	640-1400	0.9
OPB-04	GGACTGGAGT	12	12	12	0	320-2500	1.0
OPB-05	TGCGCCCTTC	12	12	12	0	460-2000	1.0
OPB-06	TGCTCTGCCC	10	11	10	1	200-1300	1.0
OPB-07	GGTGACGCAG	10	11	10	1	220-1000	1.0
OPB-08	GTCCACACGG	15	15	15	0	230-2300	1.0
OPB-09	TGGGGGACTC	9	9	9	0	680-2000	1.0
OPB-10	CTGCTGGGAC	11	12	11	1	330-1300	1.0
Kit OPC							
OPC-01	TTCGAGCCAG	12	10	10	2	150-1500	0.9
OPC-02	GTGAGGCGTC	13	13	12	2	210-1900	0.9
OPC-03	GGGGGTCTTT	9	10	9	1	440-1800	0.9
OPC-04	CCGCATCTAC	4	5	4	1	280-1200	0.9
OPC-05	GATGACCGCC	17	18	17	1	250-1700	1.0
OPC-06	GAACGGACTC	6	8	6	2	210-2500	0.9
OPC-07	GTCCCGACGA	5	6	5	1	400-1200	0.9
OPC-08	TGGACCGGTG	16	16	15	2	240-1900	0.9
OPC-09	CTCACCGTCC	10	12	10	2	440-2000	0.9
OPC-10	TGTCTGGGTG	5	7	5	2	200-1100	0.8
Kit OPD							
OPD-01	ACCGCGAAGG	7	7	7	0	650-1600	1.0
OPD-02	GGACCCAACC	7	7	7	0	320-1100	1.0
OPD-03	GTCGCCGTCA	13	13	13	0	390-1300	1.0
OPD-04	TCTGGTGAGG	6	8	6	2	510-1700	0.9
OPD-05	TGAGCGGACA	13	12	12	1	300-1600	1.0
OPD-06	ACCTGAACGG	5	5	5	0	270-1400	1.0
OPD-07	TTGGCACGGG	7	8	7	1	340-1500	0.9
OPD-08	GTGTGCCCCA	15	15	15	0	260-2300	1.0
OPD-09	CTCTGGAGAC	8	10	8	2	420-2300	0.9
OPD-10	GGTCTACACC	11	11	10	2	270-1900	0.9

4. Conclusion

This study proposed to assess the effectiveness of the vitrification method for the cryopreservation of *R. gigantea* seeds. From our results, the highest germination rate was found to be 61.33% after the seeds had been treated with a pre-culture solution containing 0.5 M sucrose, which was followed by treatment with LS solution containing 0.4 M glucose and 2 M glycerol and was then followed with dehydration in PVS2 for 30 min. Moreover, given that they have a lower moisture content than some other orchid species and/or orchid tissues, the seeds of *R. gigantea* are suitable for cryopreservation.

In order to assess the genetic stability of cryopreserved seeds, RAPD was determined to be an important tool. The results revealed that there had been no genetic alterations between the control and the cryopreserved plants. Hence, vitrification is a reliable and efficient technique that can be utilized to cryopreserve the seeds of *R. gigantea*. In order to ensure the genetic stability of the cryopreserved plants, a biochemical stability assessment should be performed in order to implement this vitrification method.

5. Acknowledgements

The authors would like to thank The Department of Biology of the Faculty of Science at Khon Kaen University for the laboratory facilities. This research was financially supported by The Science Achievement Scholarship of Thailand (SAST) and the Salt-Tolerant Rice Research Group at the Department of Biology of Khon Kaen University.

6. References

[1] Arditti J. Fundamentals of Orchid Biology. New York: Wiley; 1992.
[2] Dressler RL. Phylogeny and Classification of the Orchid Family. Melbourne: Press Syndicate of the University of Cambridge; 1993.
[3] Thammasiri K. Thai orchid genetic resources and their improvement. Horticulturae. 2016;2(3):9.
[4] Nikishina TV, Popova EV, Vakhrameeva MG, Varlygina TI, Kolomeitseva GL, Burov AV, et al. Cryopreservation of seeds and protocorms of rare template orchids. Russ J Plant Physiol. 2007;54(1):121-127.
[5] Hirano T, Godo T, Mii M, Ishikawa K. Cryopreservation of immature seeds of *Bletilla striata* by vitrification. Plant Cell Rep. 2005;23:534-539.
[6] Vendrame W, Faria RT, Surace M, Sahyun SA. Orchid cryopreservation. Ciênc agrotec. 2014;38(3):213-229.
[7] Surenciski MR, Flachsland EA, Terada G, Mroginski LA, Rey HY. Cryopreservation of *Cyrtopodium hatschbachii* Pabst (Orchidaceae) immature seeds by encapsulation-dehydration. Biocell. 2012;36(1):31-36.
[8] Worrachottiyanon W, Bunnag S. Cryopreservation of *Cymbidium finlaysonianum* Lindl. by encapsulation-dehydration method. Songklanakarin J Sci Technol. 2018;40(3):682-691.
[9] Antony JJJ, Poobathy R, Danial M, Sinniah UR, Subramaniam S. Polymorphism analysis of cryopreserved *Dendrobium* Bobby Messina protocorm-like bodies (PLBs) using RAPD markers. POJ. 2012;5(5):427-431.
[10] Kalaiselvi R, Rajasekar M, Gomathi S. Cryopreservation of plant materials-a review. Int J Chem Stud. 2017;5(5):560-564.
[11] Uragami A, Sakai A, Nagai M, Takahashi T. Survival of cultured cells and somatic embryos of *Asparagus officinalis* cryopreserved by vitrification. Plant Cell Rep. 1989;8(7):418-421.
[12] Sakai A, Kobayashi S, Oiyama I. Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb. var *Brasiliensis* Tanaka) by vitrification. Plant Cell Rep. 1990;9(1):30-33.
[13] Vendrame WA, Carvalho VS, Dias JMM. In vitro germination and seedling development of cryopreserved *Dendrobium* hybrid mature seeds. Sci Hortic. 2007;114:188-193.
[14] Tokuhara K, Mii M. Micropropagation of *Phalaenopsis* and *Doritaenopsis* by culturing shoot tips of flower stalk buds. Plant Cell Rep. 1993;13:7-11.
[15] Nishizawa S, Sakai A, Amano Y, Matsuzawa T. Cryopreservation of asparagus (*Asparagus officinalis* L.) embryogenic suspension cells and subsequent plant regeneration by vitrification. Plant Science. 1993;91:67-73.
[16] Matsumoto T, Sakai A, Yamada K. Cryopreservation of *in vitro*-grown apical meristems of wasabi (*Wasabia japonica*) by vitrification and subsequent high plant regeneration. Plant Cell Rep. 1994;13: 442-446.
[17] Ishikawa K, Harata K, Mii M, Sakai A, Yoshimatsu K, Shimomura K. Cryopreservation of zygotic embryos of a Japanese terrestrial orchid (*Bletilla striata*) by vitrification. Plant Cell Rep. 1997;16:754-757.

[18] Poobathy R, Xavier R, Sinniah UR, Subramaniam S. Molecular stability of protocorm-like bodies of *Dendrobium* Sonia-28 after encapsulation-dehydration and vitrification. Aust J Crop Sci. 2013;7(2):189-195.
[19] Jitsopakul N, Thammasiri K, Yukawa T, Ishikawa K. Effect of cryopreservation on seed germination and protocorm development of *Vanda tricolor*. ScienceAsia. 2012;38:244-249.
[20] Thammasiri K, Soamkul L. Cryopreservation of *Vanda coerulea* Griff. Ex Lindl. seeds by vitrification. ScienceAsia. 2007;33:223-227.
[21] Galdiano Jr RF, Lemos EGM, Vendrame WA. Cryopreservation, early seedling development, and genetic stability of *Oncidium flexuosum* Sims. Plant Cell Tissue Organ. 2013;114:139-148.
[22] Ferrari EAP, Colombo RC, Faria RT, Takane RJ. Cryopreservation of seeds of *Encholirium spectabile* Martius ex Schultes f. by the vitrification method. Rev Ciênc Agron. 2016;47(1):172-177.
[23] Kauth PJ, Dutra D, Johnson TR, Stewart SL, Kane ME, Vendrame W. Techniques and applications of *in vitro* orchid seed germination. USA: Global Science Books; 2008.
[24] Hirano T, Godo T, Miyoshi K, Ishikawa K, Ishikawa M, Mii M. Cryopreservation and low-temperature storage of seeds of *Phaius tankervilleae*. Plant Biotechnol Rep. 2009;3:103-109.
[25] Hu WH, Yang YH, Liaw SL, Chang C. Cryopreservation the seeds of a Taiwanese terrestrial orchid, *Bletilla formosana* (Hayata) Schltr. by vitrification. Bot Stud. 2013;54:33.
[26] Dowling N, Jusaitis M. Asymbiotic *in vitro* germination and seed quality assessment of Australian terrestrial orchids. Aust J Bot. 2012;60:592-601.
[27] Uragami A, Sakai A, Nakai M. Cryopreservation of dried axillary buds from plantlets of *Asparagus offinalis* L. grown in vitro. Plant Cell Rep. 1990;9:328-331.
[28] Butiuc-Keul A, Farkas A, Cristea V. Genetic stability assessment of *in vitro* plants by molecular markers. Stud Univ Babes Bolyai Biol. 2016;1:107-114.