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Original Article

A simple and reproducible protocol for plant regeneration and cryopreservation of *Grammatophyllum specinocum* BL.

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Abstract

Grammatophyllum specinocum BL. is native to Thailand and is at risk of extinction due to human-caused environmental changes and commercial exploitation. Cryopreservation is an efficient approach for conservation of plant germplasms. The aim of this study was to establish a simple and reproducible protocol for callus induction and plant regeneration, and cryopreservation of *Grammatophyllum specinocum* BL. The orchid seeds successfully germinated on half-strength Murashige and Skoog (½ MS) medium. Amendment of 0.5 mg/l 6-benzylaminopurine (6-BA) into MS medium was suitable for callus induction whilst a combination of 1 mg/l 6-BA and 2 mg/l naphthaleneacetic acid (NAA) was effective in promoting plantlet regeneration. Protocorms of *G. specinocum* were successfully cryopreserved using encapsulation-dehydration method with the following protocol: 24-h preculture of protocorms with 0.75 M sucrose solution in the dark followed by encapsulation of the precultured protocorms in Ca-alginate beads, 24-h pretreatment of encapsulated beads with 0.5 M sucrose solution in the dark, and 8-h dehydration before plunging into liquid nitrogen. Random amplified polymorphic DNA (RAPD) analysis was carried out to detect genetic stability of cryopreserved protocorms and genetic differences not detected between non-cryopreserved and cryopreserved protocorms.

Keywords: cryopreservation, plant regeneration, orchid, RAPD

1. Introduction

Orchidaceae is the largest family of the phylum of Angiosperm, commonly known as a group of flowering plants (Pradhan *et al.,* 2014; Weston *et al.,* 2005). Apart from their immense horticultural and medicinal importance, orchids play a very useful role in the balance of the forest ecosystems (Pradhan *et al.,* 2014). As cut flowers or as potted plants, orchids occupy top position with a high return in the international market (Galdiano Jr. *et al.,* 2012). The orchid business has been reported to cover around 8% of the world floriculture trade (Chugh *et al.,* 2009), with the trade value observed to reach US$504 million during 2007-2012 (Cheamuangphan *et al.,* 2013). Orchids are terrestrial herbaceous perennials that have a symbiotic relationship with mycorrhizal fungi to drive energy throughout a portion or all of their life cycle (Rasmussen, 1995), especially for seed germination and nutritional support until the plants reach a level of autotrophy (Sharma *et al.,* 2003). Despite a very large number of seeds, only a few seeds germinate in nature as they lack food reserves and require a specific fungus (Kalimuthu *et al.,* 2007; Steinfort *et al.,* 2010), thus making them vulnerable to extinction. Nowadays the conservation of orchids is becoming a matter of global concern due to the alarming decline in their natural populations, and the Orchidaceae on the whole are now included in the Appendix-II of CITES (Senthilkumar, 2001). The orchid diversity in Northeast Thailand and the country as a whole is being threatened by various factors such as deforestation, habitat destruction, and overexploitation. *Grammatophyllum specinocum* BL, which is native to the north and northeast regions of Thailand, is recognized as one of the threatened orchid species that require urgent conservation measures.

Cryopreservation plays a pivotal role in order to formulate sustainable conservation of rare, endangered, and threatened plants (Thammasiri, 2005). This technology is one

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of the most acceptable methods for long-term storage of living materials at an ultra-low temperature (-196 ºC) since almost all biological activities of cells ceased immediately under this condition (Engelmann, 2011; Sakai, 2004). However, exposed to such low temperatures without any pretreatment biological specimens are usually damaged because of intracellular freezing (Hirano *et al.,* 2009). Several pretreatments of cryopreservation including various desiccation methods and vitrification have been evaluated for their efficiency on viability and germination rates of a variety of orchid species (Bian *et al.,* 2002; Galdiano Jr *et al.,* 2012; Huehne & Bhinija, 2012), and encapsulation-dehydration was reported as a simple and promising method for successful cryopreservation (Bunnag & Hongthongkham, 2014; Surenciski *et al.,* 2012; Yin *et al.,* 2011).

In this study, the conditions for cryopreservation of protocorms of *G. specinocum* were optimized. In vitro germination, protocorm recovery, and seedling development of cryopreserved protocorms were assessed. Genetic stability of cryopreserved protocorms was also assessed by the random amplified polymorphic DNA (RAPD) technique on account of the fact that explants exposed to very low temperatures can possibly come up with genetic instability (Antony *et al.,* 2012).

2. Materials and Methods

2.1 Plant material and culture conditions

Grammatophyllum specinocum BL. was examined for its viability for cryopreservation. Mature orchid pods were freshly harvested and sterilized by soaking in 95% (v/v) ethanol. The excess alcohol was carefully flamed off the pods three times. After scraping out the seeds, they were examined for their germination and protocorm development percentages on semi-solid Murashige and Skoog (MS) medium and half-strength semi-solid MS (½ MS) medium (Murashige & Skoog, 1962) containing 8% agar as the gelling agent. Unless otherwise stated, the pH of the culture media was adjusted to 5.7 before sterilization by autoclaving at 121 ºC for 20 min. Plants were cultured under standard conditions: 16-h photoperiod with cool white light (3000 lx) provided by Philips cool-white fluorescent lamps, at 25 ± 2 ºC. To examine the effect of plant growth regulators on protocorm proliferation, 8-week-old protocorms were further cultured for 8 weeks on MS medium amended with 6-benzylaminopurine (6-BA) at concentrations of 0.5, 1, 1.5, 2, and 2.5 mg/l. Then the obtained protocorms were transferred to semi-solid ½ MS medium plus a combination of naphthaleneacetic acid (NAA; 1, 2, and 3 mg/l) and 6-BA (0.5 and 1 mg/l) and cultured for 24 weeks to find the optimal conditions for plant regeneration. Subculture was performed at four-week intervals.

2.2 Pretreatment, encapsulation and cryopreservative experiments

Five-week-old protocorms were precultured in 0.75 M sucrose solution for 24 h in the dark. Encapsulation was carried out by soaking the precultured protocorms in 2.5% Na-alginate solution prepared in Ca-free liquid ½ MS medium. The encapsulated beads were then formed in 0.1 M $CaCl_2$ solution and allowed to settle for 30 min. Following settlement, the obtained beads were rinsed twice with liquid ½ MS medium.

Prior to dehydration, the beads were pretreated with liquid ½ MS medium containing 0.5 M sucrose for 24 h in the dark. To determine the optimal dehydration time, the pretreated beads were air-dried at ambient temperature in a laminar air-flow cabinet for 0, 2, 4, 6, 8, and 10 h. The dehydrated beads were loaded into cryotubes and plunged directly into liquid nitrogen for 1 d. To evaluate the efficacy of the cryopreservation protocol, the pretreated beads were air-dried for 8 h and immersed into liquid nitrogen for 1, 2, and 3 weeks. At the given timepoints, the cryotubes were taken out from the liquid nitrogen and rapidly warmed in a waterbath at 38 ºC for 2 min. Then the cryopreserved protocorms were transferred into liquid ½ MS medium containing 0.25 M sucrose for 20 min and washed three times with sterile distilled water prior to regrowth on semi-solid MS medium.

2.3 Experimental designs

A completely randomized design with three replicates and ten protocorms per replicate was used to determine the effect of cryopreservation on orchid growth and genetic stability. Water content was examined at 2-h intervals. The viability of the protocorms was assessed three weeks after treatment based on the 2, 3, 5-triphenyl tetrazolium chloride (TTC) histochemical assay and regrowth was based on the number of regenerated protocorms. Genetic stability of the cryopreserved and non-cryopreserved protocorms was based on the RAPD analysis using the RAPD 10mer Kit (Eurofins, Louisville, KY, USA) (Table 1). The reaction components are listed in Table 2 and the thermal cycling conditions are presented in Table 3. The results are expressed as mean ± one standard error (SE) of three replicates and data were analyzed using one-way analysis of variance with Duncan's multiple range test to determine the significance relative to the control. In all cases, $P<0.05$ was considered significant.

Table 1. RAPD primers.

Primer	Nucleotide sequence (5'-------3')
OPA01	GGTGACGCAG
OPA05	ACGCCAGAGG
OPA06	CAGGGGTGGA
0PB02	TGATCCCTGG
OPB04	GGACTGGAGT
OPB09	TGGGGGACTC
OPB15	GGAGGGTGTT
OPB20	GGACCCTTAC
OPC03	GGGGGTCTTT
OPC04	CCGCATATAC
0PC10	TGTCTGGGTG
OPX01	CTGGGCACGA
0PX06	ACGCCAGAGG
0PX08	CAGGGGTGGA
OPX09	GGTCTGGTTG
OPX17	GACACGGACC
0PBH05	GTAGGTCGCA
0PBH19	GTCGTGCGGA

Table 2. Reaction components for PCR.

Components	Amount (µl)
dH_2O	14.45
10X buffer *Taq* DNA polymerase	2
dNTP (10 mM)	0.4
$MgCl_2$ (25 mM)	1.2
Primer (10 µM)	1
Taq DNA polymerase (5 units/µl)	0.25
DNA template	0.7

Table 3. RAPD thermal cycling conditions.

Step	Temperature (ºC)	Time	Cycle
Initial denature	93	4	1
Denature	93	30	30
Annealing	37	30	30
Extension	72	45	30
Final extension	72	5	1
Hold	4	-	-

3. Results and Discussion

3.1 Germination, protocorm induction and plant regeneration

Two culture media (MS and ½ MS) were tested for their suitability for the orchid seed germination. It was evident that the orchid seeds successfully germinated on both MS medium and ½ MS medium with their differences in germination rates (Figure 1). The orchid seeds showed a germination rate of 90 and 70% on ½ MS medium and MS medium, respectively. After a 45-day germination period, large-sized protocorms with a dark green color were observed on the ½ MS medium while small-sized protocorms with a pale green color were seen on the MS medium. Therefore, the ½ MS medium was chosen for subsequent experiments. It is well-documented that other than seed maturity (Zhang *et al.,* 2013) culture media and growth conditions play a crucial role in the orchid seed germination. Even for the same species, there have been reports of different culture media giving better conditions for germination (Chen *et al.,* 2004; Ding *et al.,* 2004).

The initiation and proliferation explants are largely influenced by external addition of plant growth regulators. In this study, the obtained protocorms were cultured on semi-solid ½ MS medium fortified with various concentrations of 6-BA (0.5, 1, 1.5, 2, and 2.5 mg/l) in order to find the appropriate concentration of 6-BA that promoted protocorm enlargement and proliferation. Without the amendment of 6-BA (control groups), protocorm enlargement was observed with no sign of proliferation. On the other hand, addition of 6-BA to the media exerted protocorm enlargement and proliferation (Figure 2). 6-BA at concentrations of 0.5, 1, 1.5, 2, and 2.5 mg/l were observed to increase the total protocorm weight by 0.31, 0.28, 0.19, 0.12, and 0.24 g, respectively. Thus, 6-BA at a concentration of 0.5 mg/l was suitable for protocorm proliferation. In most of the orchids, cytokinins applied either singly or in combination with auxins have been demonstrated to induce protocorm production (Bhattacharyya *et al.,* 2013; Dohling *et al.,* 2012; Zhao *et al.,* 2008). However, the results obtained in this study indicated that 6-BA was suitable to be used as a substitute for auxin-cytokinin combination as described elsewhere (Kishor & Devi, 2009; Malabadi *et al.,* 2005).

To produce plantlets, a combination of auxins and cytokinins is required. In this study, the protocorms were cultured on semi-solid ½ MS medium plus 6-BA (0.5 and 1 mg/l) and NAA (1, 2, and 3 mg/l). It was observed that all treatments, including the controls, were suitable for inducing plant regeneration (Figure 3). In the control groups, the viability of the protocorms was 73.33% and the obtained plants had the height of 1.43 cm and the median root number of 1.62 (Figure 4A). Meanwhile, a combination of 1 mg/l 6-BA and 2 mg/l NAA was found to be most effective for plant regeneration. With this treatment, the viability of the protocorms was 73.33% (Figure 4B) and the obtained plants showed the average height and root number of 2.5 cm and 2.63, respectively.

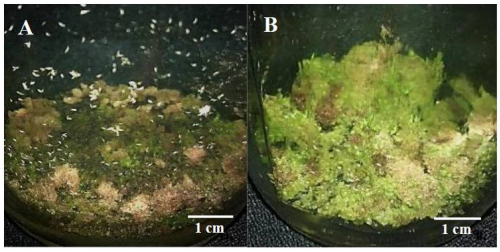

Figure 1. Characteristics of protocorms obtained following 45 days of seed germination on (A) MS medium and (B) ½ MS medium.

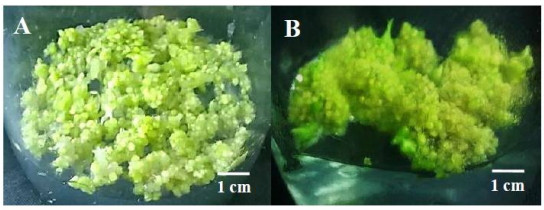

Figure 2. Characteristics of protocorms cultured on proliferation media for 8 weeks. (A) ½ MS medium without 6-BA added. (B) ½ MS medium plus 0.5 mg/l 6-BA.

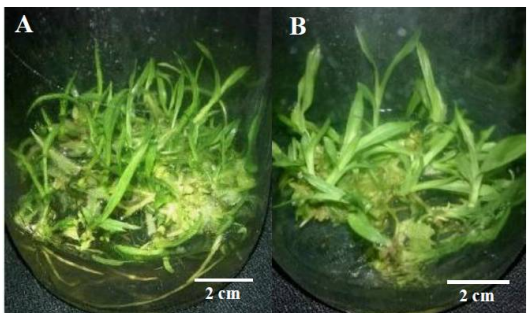

Figure 3. Sixteen-week-old plants raised from seed-derived protocorms cultured o n (A) ½ MS medium without 6-BA and NAA amended and (B) ½ MS medium plus 1 mg/l 6-BA and 2 mg/l

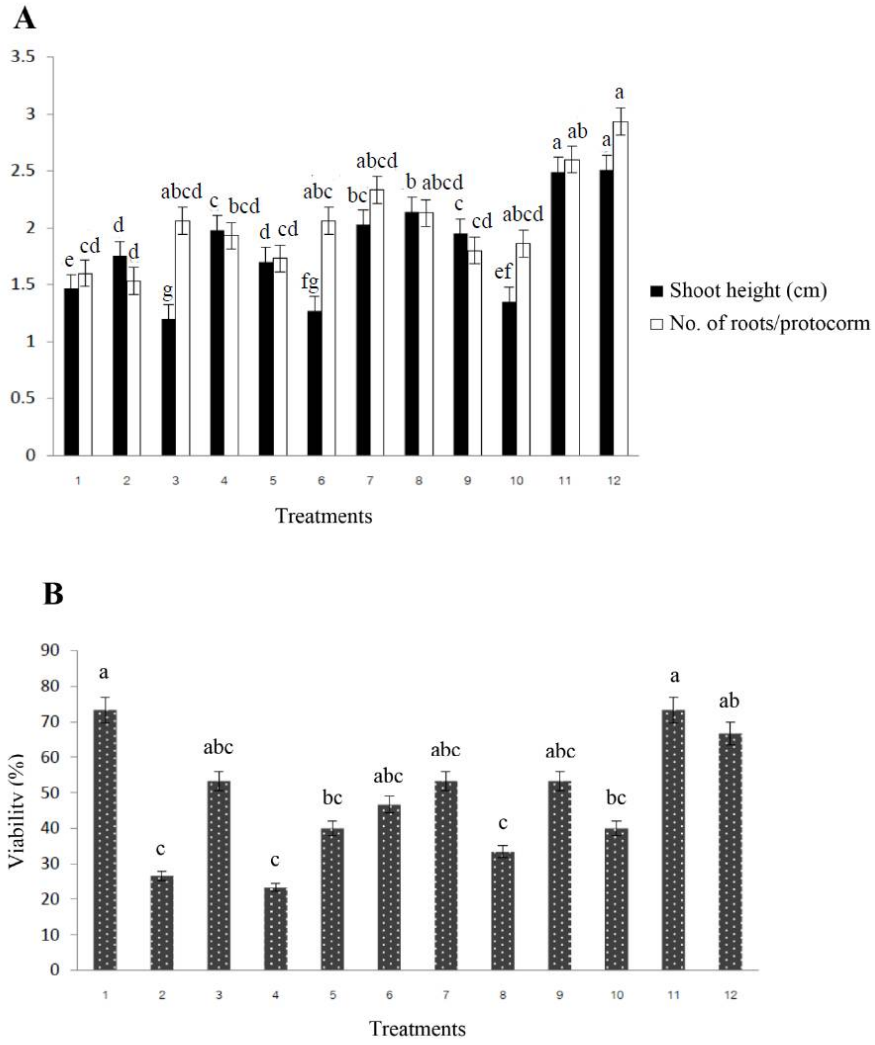

Figure 4. (A) Shoot height (in cm) and mean numbers of roots per protocorm recorded after 24 weeks of culture. (B) Percent viability of protocorms cultured for 16 weeks on ½ MS medium plus 6-BA and NAA at various concentrations: **1**, ½ MS; **2**, ½ MS + 1 mg/l NAA; **3**, ½ MS + 2 mg/l NAA; **4**, ½ MS + 3 mg/l NAA; **5**, ½ MS + 0.5 mg/l 6-BA; **6**, ½ MS + 0.5 mg/l 6-BA + 1 mg/l NAA; **7**, ½ MS + 0.5 mg/L 6-BA + 2 mg/l NAA; **8**, ½ MS + 0.5 mg/L 6-BA + 3 mg/l NAA; **9**, ½ MS + 1 mg/l 6-BA; **10**, ½ MS + 1 mg/l 6-BA + 1 mg/l NAA; **11**, ½ MS + 1 mg/l 6-BA + 2 mg/l NAA; **12**, ½ MS + 1 mg/L 6-BA + 3 mg/l NAA. Bars represent standard error.

3.2 Water content, viability and regrowth

In this study, cryopreservation of the orchid protocorms was based on an encapsulation-dehydration method. After a 24-h preculture in 0.75 M sucrose solution in the dark, the precultured protocorms were encapsulated in Ca-alginate. The resulting protocorms were pretreated with 0.5 M sucrose for 24 h in the dark and air-dried for 0-10 h before plunging into liquid nitrogen to determine water content as well as the protocorm viability. As expected, pretreatment with 0.5 M sucrose was insufficient to reduce the protocorm water content since only an 8% decrease was observed. Meanwhile, air drying caused a profound decrease in the protocorm water contents. When subjected to air-drying for 2, 4, 6, 8, and 10 h, the protocorm water contents decreased from 92 to 84, 79, 47, 16, and 9%, respectively (Figure 5A). After air-drying, the protocorms were plunged into liquid nitrogen for 1 d to assess the effect of water content on the viability of protocorms during cryopreservation. It was observed that the water contents of 84, 79, 47, 16, and 9% gave rise to protocorm viabilities of 0, 4, 8, 20, and 8%, respectively as assessed by the TTC histochemical assay (Figure 5B). According to the results, an air-drying time of 8 h was the most effective and allowed the highest viability of protocorms. To determine the effectiveness of the encapsulation-dehydration method employed in this study, the protocorms were submerged into liquid nitrogen for 1, 2, and 3 weeks and the results are given in Figure 6A. It was found that the viability of the cryopreserved protocorms reduced according to the prolonged cryopreservation time as evaluated by the TTC histochemical

assay. The protocorm viabilities were 20, 12, and 8% when the cryopreservation time was extended to 1, 2, and 3 weeks, respectively. However, there were no significant differences among the treatments. After 1-3 weeks of cryopreservation, the cryopreserved protocorms were tested for their capability of regrowth on semi-solid half-strength MS culture media containing 1 mg/l 6-BA and 2 mg/l NAA. It was noted that the 1-, 2-, and 3-week cryopreserved protocorms showed viabilities of 16, 16, and 12%, respectively (Figure 6B). Also, the 1-, 2-, and 3-week cryopreserved protocorms showed the capability of regrowth of 8, 4, and 4%, respectively (Figure 6C).

In general, the encapsulation-dehydration technique used for cryopreservation of plant germplasms involves the formation of alginate beads, followed by osmotic dehydration in concentrated sucrose solution and further exposure to air-drying, prior to plunging the samples into liquid nitrogen (Khoddamzadeh *et al.,* 2011; Surenciski *et al.,* 2012; Yin *et al.,* 2011; Zalewska & Kulus, 2014). The overall objective of such pretreatments is to produce beads with water as little as possible in order to minimize or prevent ice formation during cryogenic storage (Trigiano & Gray, 2011), thereby allowing vitrification as well as increasing survival post-cryostorage. As more water in the beads becomes unfrozen or vitrified upon cooling, the greater is likely to be the level of the sample viability. However, the thermal properties conferred on the alginate beads during their preparation also play a significant role in the success of the pretreatments and hence, the survival of the plant sample after cryogenic storage.

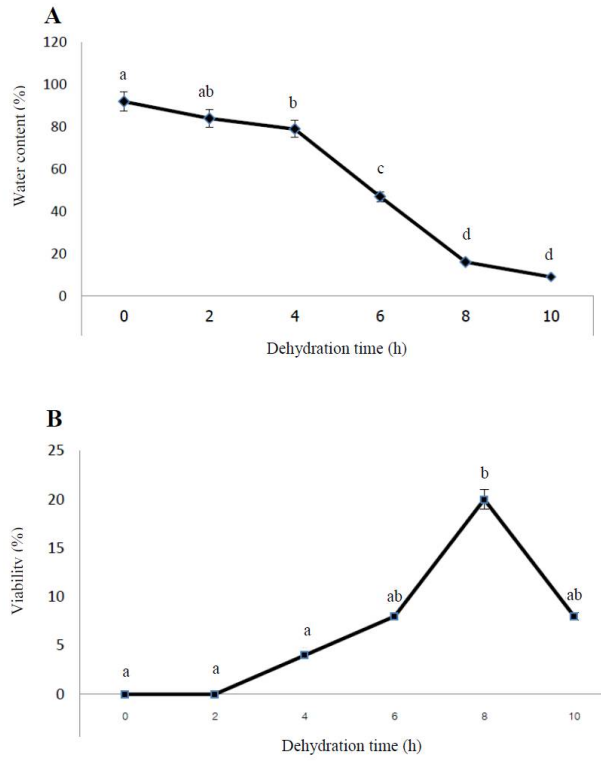

Figure 5. (A) Water contents of 0.5 M sucrose-pretreated protocorms subjected to air-drying for 0-10 h. (B) Percent viability of the 1-day cryopreserved protocorms previously pretreated with 0.5 M sucrose and air-dried for 0-10 h.

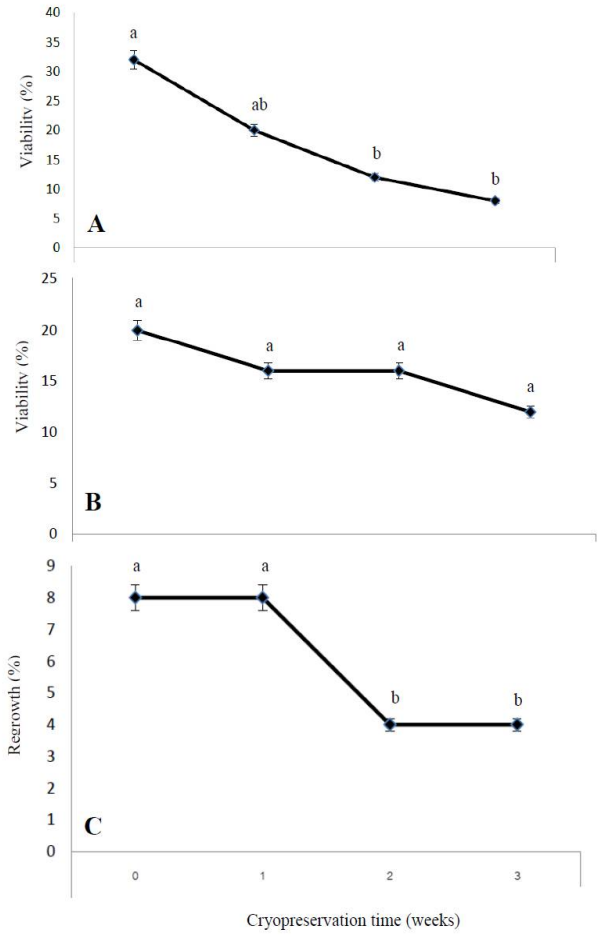

Figure 6. (A) Percent viability of the protocorms cryopreserved for 1, 2 and 3 weeks. (B) Percent viability of the cryopreserved protocorms cultured on ½ MS medium plus 1 mg/l 6-BA and 2 mg/l NAA for 3 weeks. (C) Percent regrowth of the cryopreserved protocorms.

3.3 Genetic stability

RAPD was employed to detect the clonal variations of the cryopreserved protocorms using 20 primers from Eurofins (Table 1). Both the cryopreserved and non-cryopreserved protocorms generated bands with 6 primers, including OPA05, OPA06, OPB09, OPB20, OPC03 and OPX01 (Figure 7). Clonal variations were not detected, indicating the genetic stability of the cryopreserved protocorms. RAPD markers may be among the more sensitive, simple, efficient tools for genetic diversity analysis between the cryopreserved and non-cryopreserved protocorms and effectively trace their genetic relationships. However, more primers for RAPD should be used along with the use of the other molecular markers in order to confirm the genetic stability of the cryopreserved protocorms.

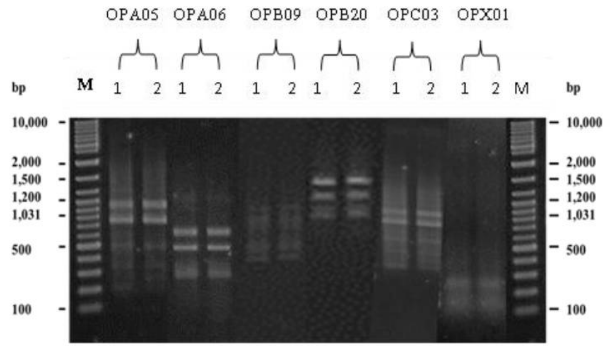

Figure 7. RAPD profiles obtained for the cryopreserved and non-cryopreserved protocorms amplified with 6 primers.

4. Conclusions

A simple and reproducible protocol for plant regeneration and cryopreservation of *Grammatophyllum specinocum* BL. has been proposed in this study. Orchid seeds germinated successfully on half-strength semi-solid MS culture media. The addition of 0.5 mg/l 6-BA to semi-solid MS culture media was sufficient to induce callus formation while a combination of 1 mg/l 6-BA and 2 mg/l NAA was found to promote plantlet regeneration. Orchid protocorms were successfully cryopreserved utilizing the encapsulation-dehydration method using these conditions: 24-h preculture of protocorms with 0.75 M sucrose solution in the dark followed by encapsulation of the precultured protocorms in Ca-alginate beads, 24-h pretreatment of encapsulated beads with 0.5 M sucrose solution in the dark, and 8-h dehydration before plunging into liquid nitrogen. RAPD analysis was carried out to detect genetic stability of cryopreserved protocorms and genetic differences between cryopreserved and non-cryopreserved protocorms were not detected.

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