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DEVELOPMENT OF CRYOPRESERVATION PROTOCOLS FOR FIVE INDIGENOUS MEDICINAL ORCHIDS OF BANGLADESH

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Abstract

The efficiency of post-thaw recovery of cryopreserved protocorms of five indigenous medicinal orchid species namely, *Acampe papillosa, Aerides odorata, Bulbophyllum lilacinum, Cymbidium aloifolium,* and *Pholidota pallida* was investigated. Regeneration rates of protocorms after post-thawed recovery widely varied from genus to genus in protocorms cryopreserved using cryoprotectant. The highest rate of recovery was 96% in *Cymbidium aloifolium* and the lowest was 86% in post-thawed protocorms of *Acampe papillosa* on MS based regeneration medium semi-solidified with 0.4% (w/v) agar and supplemented with 2 mg l-1 BAP and 3% (w/v) sucrose using cryoprotectant method. The highest rate of recovery was found to be 97% and it was lowest (89%) on the same medium using encapsulation method. Though there was no significant difference on post-thawed recovery using cryopreservation method either using cryoprotectant or encapsulation technique, the growth index of cryopreserved protocorms in case of encapsulation method was better than cryoprotectant method. The highest rate of growth index was in *Acampe papillosa* (56%) and the lowest growth index was 19% in case of *Cymbidium aloifolium*. The presently used method of development of cryopreservation protocol seems to be a promising technique for cryopreservation of some medicinal orchids of Bangladesh.

Introduction

ORCHIDACEAE IS one of the largest and most diverse family of the flowering plants. It comprises 700-800 genera and 20,000-35,000 species occurring in every habitat, except in the polar zone; 10% are endangered in their native habitats. There are currently no major government efforts to conserve orchid germplasm using seeds and in this connection, there is a need to develop procedures to improve orchid seed storage. IUCN has listed orchids as endangered species due to disturbance of their natural habitats and climate changes. The cryopreservation of wild orchid seeds is an important conservation method as seeds are heterogeneous in most wild orchids and their preservation may help conserving the genetic diversity of plant populations. However, as compared to traditional methods of storage, the method of cryopreservation is cost effective, requires little space and is efficacious for the long term storage of plant genetic resources (Engelmann, 2011). Therefore, it is rather important to develop reliable conservation protocols to conserve the endangered species in nature.

The orchid seeds are minute, microscopic and are produced in large numbers; they require suitable mycorrhizal fungus for their germination in nature. It has been suggested for the first time in 1979 that cryopreservation (storage in liquid nitrogen at -196^0C) of seeds, shoot meristems and cells *in vitro* could be used in addition to conventional methods of conservation of endangered species of plants (Tikhonova, 1999). Cryopreservation has also been touted as a practical and promising tool for long term orchid conservation (Johnston *et al.,* 2009). Cryopreservation completely inhibits all processes of cell metabolism and seed aging, hence thereby providing antibiosis for almost unlimited time. Tikhonova (1999) was the first who used cryopreservation technique for storage of seeds of rare wild plant species in 1986. The orchid explants such as protocorms and protocorm like bodies of *Dendrobium candidum* were successfully cryopreserved (Brian *et al.,* 2002). The synthetic seeds of orchids have been produced by encapsulation of protocorm-like bodies (PLBs) in an alginate gel (Kaur and Pathak, 2014; Pathak and Vij, 2005; Pehwal *et al.*, 2012; Saiprasad and Polisetty, 2003; Sembi *et al.*, 2006; Vij *et al.,* 1993).

According to Pritchard (1984), cryopreservation of orchid seeds faces significant obstacles because of methodological problems involved with determination of seed germination rate *in vitro* and selection of optimum freezing conditions. Protocorms/ protocorms-like bodies are considered as suitable explants capable of multiplication and regeneration into complete plantlets. Thus, the development of a long-term preservation method using embryos, protocorms or protocorms-like bodies is very important with a view to conserving orchid germplasm for breeding programmes and the orchid floricultural industry. However, reports regarding cryopreservation of orchid

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protocorms through encapsulation method or with cryoprotectant in liquid nitrogen or in freezing condition are not available in our country. Therefore, the present study was undertaken with a view to developing efficient and reliable *ex situ* conservation protocols for some medicinally important orchids of Bangladesh.

Materials and Methods

Plant Materials and Preparation of Protocorms

Protocorms obtained after seed germination of orchid species namely, *Acampe papillosa, Aerides odorata, Bulbophyllum lilacinum, Cymbidium aloifolium, and Pholidota pallida.* Sixty days old protocorms with the large diameter of 1-2 cm were subjected to cryopreservation. Three months old protocorms were used for experimentation. Prior to cryopreservation, protocorms were cultured for 3-4 days in liquid MS medium (Murashige and Skoog, 1962) supplemented with 60 gl^{-1} sorbitol. For encapsulation, protocorms were cultured on semi-solidified [with 0.4% (w/v) agar] MS based regeneration medium supplemented with 2 mg l^{-1} BAP and 3% (w/v) sucrose for 30 days.

Preparation of Cryoprotectant Solution

To prepare cryoprotectant, protocol was followed as described by Lynch *et al.* (1994). The cryoprotectant solution was prepared by adding Glycerol 4.6 ml/100 ml, DMSO (dimethylsulphoxide) 3.9 ml/100 ml, Sucrose 34.2 g/100 ml and Proline 0.5 g/l00 ml. The cryoprotectant mixture was dissolved in final volume of 100 ml MS-based liquid medium and the pH was adjusted to 5.8 prior to sterilization. After sterilization, cryoprotectant solution was stored in refrigerator.

Cryopreservation of Protocorms by Using Cryoprotectant

The cryopreservation of protocorms using cryoprotectant was based on that of Meijer *et al.* (1991). Sixty days old protocorms with the length of 3-5 mm were harvested and placed into the 3 cm^3 glass vials and approximately 1-1.5 ml of cryoprotectant solution was taken to each cryovial. Then cryovials (Figs. 1a,b) were sealed and placed in ice for 1 hr and subsequently, stored at -20^{0}C in freezer.

Preparation of Solution for Encapsulation

The solution of 100 mM $Ca(NO_3)_2$ was prepared by adding 23.62 g of $Ca(NO_3)_2.4H_2O$ in one liter of distilled water. The $Ca(NO_3)_2$ solution was mixed until dissolved; it was poured into a one liter bottle, the bottle was then capped and sterilized. A 2% alginate solution was also prepared in MS liquid medium. Bottles containing $Ca(NO_3)_2$ solution and the flasks containing 2% sodium alginate were autoclaved for 20 min and allowed to cool for at least 1 hr. These were subsequently stored in freezer at 4° C for 3-4 days.

Encapsulation of Protocorms by Alginate Gel

The procedures were performed using sterile technique inside a sterile laminar-airflow hood. Approximately 100 ml of sterile $Ca(NO_3)_2$ salt solution was poured into a sterile 250 ml beaker. Protocorms were mixed with alginate solution properly. Alginate solution with protocorms was then slowly dropped from the cut pipette that was positioned above the $Ca(NO_3)_2$ salt solution and slowly beads were formed (Figs. 1c, d). The alginate beads which encapsulated protocorms were kept in $Ca(NO_3)_2$ enriched liquid nutrient medium for 20 min. After that the solution was removed and the beads were then washed with distilled water with gentle shaking for 20 min to remove excess $Ca(NO_3)_2$ solution.

Cryopreservation and Plant Regeneration from Post Thawed Encapsulated Protocorms

The beads containing protocorms were incubated in MS liquid protocorms medium containing 2 M glycerol and 0.4 M sucrose with gentle shaking at 25°C. Three protocorms were transferred to a 0.5 ml cryovial with 0.3 ml of the cryoprotectant solution. The cryovials were placed in a laboratory freezer at –32°C. After more than 60 days of storage, the vials were then taken out from the freezer and warmed in a water bath at 40°C. To dilute the cryoprotectant solution, the beads were incubated in MS liquid medium containing 1.2 M sucrose with gentle shaking for 15 min at 25°C. After these beads were cultured on 3% (w/v) agar semi-solidified MS-based medium supplemented with 2 mg l^{-1} BAP + 3% (w/v) sucrose and incubated in light condition for 60 days.

Recovery of Post-thawed Cryopreserved Protocorms in Cryoprotectant Solution

Protocorms were thawed by plunging the vials into sterile water at 45°C for 3-4 min. Excess cryoprotectant was removed under aseptic conditions from the protocorms using a Pasteur pipette. The protocorms from one vial were placed onto two superimposed filter paper discs on 20 ml aliquots of MS medium supplemented with 2 mg l^{-1} BAP + 3% (w/v) sucrose and 0.4 % (w/v) agar in test tube/Petri dishes and incubated at 26 ± 2°C in the light condition for 60 days.

Results and Discussion

Presently the protocorms were cryopreserved using cryoprotectant and encapsulation method with a view to developing a reliable approach for *ex situ* preservation of indigenous orchids of Bangladesh. Table 1 and 2 represent percentage of recovery of post-thawed protocorms. In the Table 1, the percentage of recovery of post-thawed protocorms widely **v**aried from species to species on MS based regeneration medium supplemented with 2 mg l^{-1} BAP (Figs. 2a,b). The highest rate (96%) of recovery was in *Cymbidium aloifolium* whereas it was lowest (86%) in case of *Acampe papillosa*. The rate of recovery was 94% in *Bulbophyllum lilacinum* (Table 1) and it was 93% in post-thawed protocorms of *Pholidota pallida*. To 2014. Therefore, it is suggested that by applying both the protocols protocorms of orchids can be preserved for long time.

In case of cryopreservation of protocorms by encapsulation method, the data regarding post-thawed recovery of cryopreserved protocorms (Table 2) showed that it was not significantly reduced. The percentage of recovery varied from species to species. The lowest rate of recovery was 89% in *Acampe papillosa* and *Aerides odorata* and the highest rate of recovery was 97% in *Cymbidium aloifolium.* Table 2 showed the percentage of recovery of post-thawed protocorms was 91% in *Pholidota pallida* and 90% in *Bulbophyllum lilacinum*. It was observed that *Cymbidium aloifolium* comparatively showed better

Table 1. Effect of cryopreservation using cryoprotectant on post-thawed recovery of cryopreserved protocorms.

Species	Number of post-thawed protocorms inoculated on MS medium	Number of germinated protocorms on MS medium	Recovery of post-thawed protocorms (%)
Acampe papillosa	70	60	86
Aerides odorata	90	85	95
Bulbophyllum lilacinum	80	75	94
Cymbidium aloifolium	90	85	96
Pholidota pallida	70	65	93

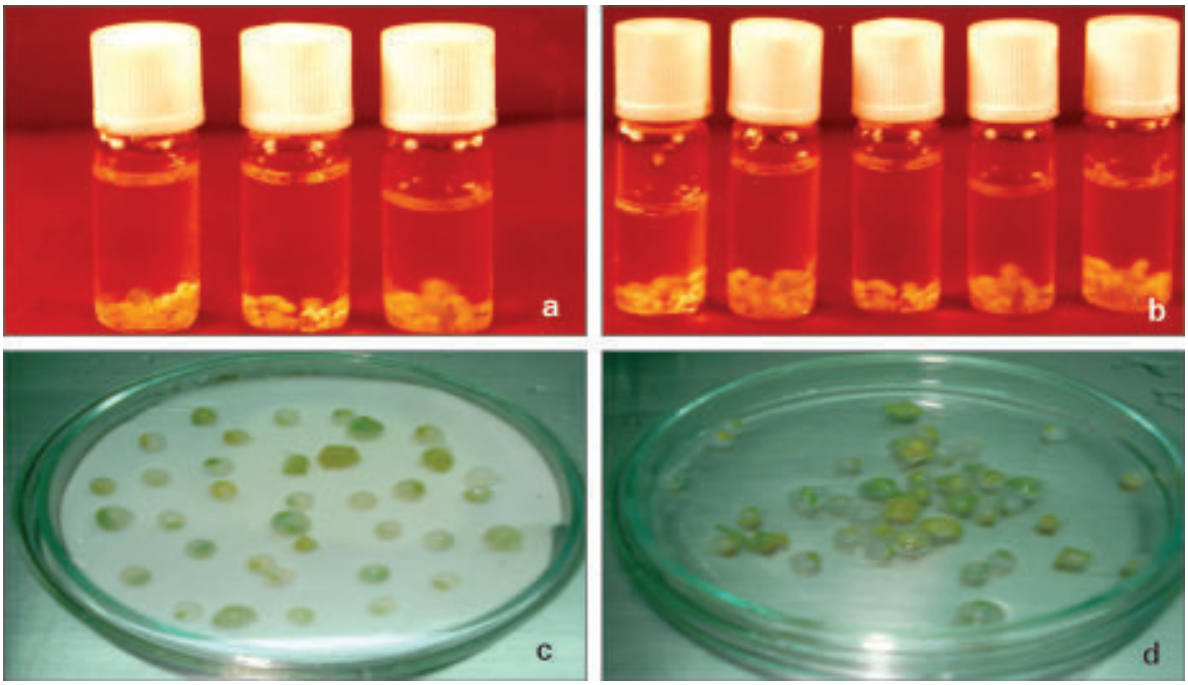

Fig. 1 a-d. Two types of cryopreservation methods: a-b, Protocorms with cryoprotectant solution into cryovials (*Acampe papillosa, Cymbidium aloifolium*); c-d, Encapsulated protocorms (*Acampe papillosa, Cymbidium aloifolium*).

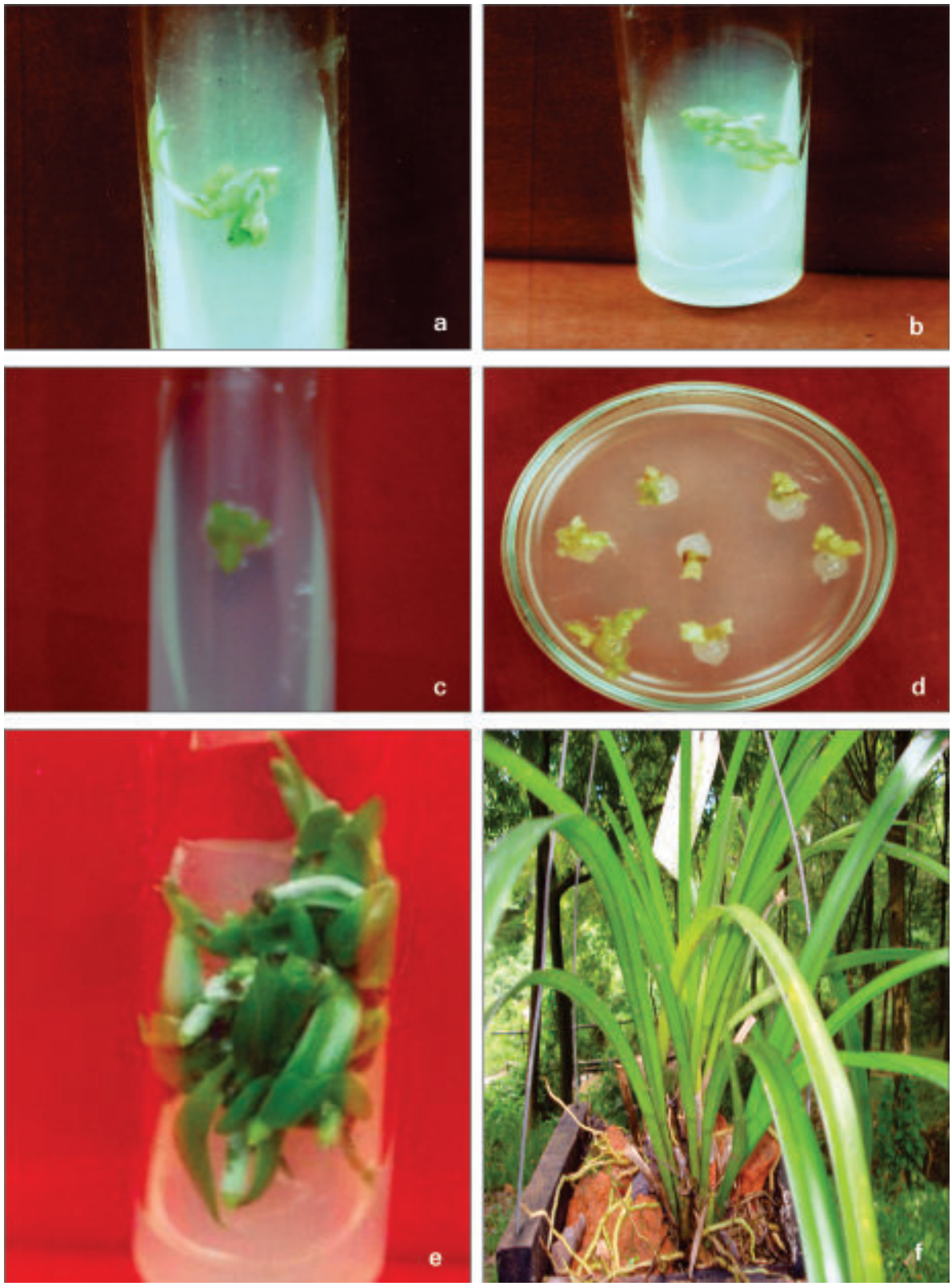

Fig. 2 a-f. Germination of cryopreserved protocorms after post-thawed recovery from two cryopreservation methods: a-b, Germination of cryoprotectant protocorms (*Cymbidium aloifolium, Pholidota pallida*); c-d, Germination of encapsulated protocorms (*Acampe papillosa, Bulbophyllum lilacinum*); e, Elongation of germinated protocorms from encapsulated cryopreserved protocorms (*Cymbidium aloifolium*); f, *In vitro* developed plants in natural condition (*Cymbidium aloifolium*).

Table 2. Effect of cryopreservation by encapsulation method on post-thawed recovery of cryopreserved protocorms.

Species	Number of post-thawed protocorms inoculated on MS medium	Number of germinated protocorms on MS medium	Recovery of post-thawed protocorms (%)
Acampe papillosa	70	62	89
Aerides odorata	90	80	89
Bulbophyllum lilacinum	80	72	90
Cymbidium aloifolium	90	87	97
Pholidota pallida	70	64	91

recovery in encapsulation method after post-thawed recovery of cryopreserved protocorms cultured on MS based regeneration medium supplemented with 2 mg l^{-1} BAP (Figs. 2c,d).

The comparative studies of growth index of post-thawed cultured protocorms from cryoprotectant and encapsulation method are presented in Table 3. The results revealed that growth index of cryopreserved protocorms in case of encapsulation method was better than in cryoprotectant method on MS based regeneration medium supplemented with 2 mg l^{-1} BAP (Fig. 2e). The increased growth rate varied from 19-56% in encapsulation method over cryoprotectant method. The lowest growth index was 19% in case of *Cymbidium aloifolium*. *In vitro* regenerated plants of *C. aloifolium* from cryopreserved protocorms were transferred to natural conditions (Fig. 2f). The comparative results between cryoprotectant and encapsulation method on growth index of post-thawed cultured protocorms demonstrated that the growth rate was different in two presently tested methods (Table 3). The growth rate reduced by freezing and thawing can be probably explained by damage of the testa (Nikishina *et al.,* 2001; Mauro *et al.*, 2012). Obviously, the nutrient-medium accessibility to the embryo through a damaged testa is higher than through an intact one where this finding is an agreement with Nikishina *et al.*, 2001. A mixture of 2.0 M glycerol and 0.4 M sucrose was reported to be very effective in inducing dehydration and freezing tolerance in various cells and meristems (Matsumoto *et al.,* 1994; Sakai *et al.,* 1991a). The loading solution contributes to minimizing the injurious membrane changes resulting from severe dehydration. Preculturing with a high concentration of sugar was also reported to be very important in improving survival of cryopreserved cells and meristem (Matsumoto *et al.*, 1994). The exact mechanism by which preculturing increases the dehydration tolerance is not known. Pre-culture results in the accumulation of sugars may increase the stability of membranes under severe dehydration conditions (Crowe *et al.,* 1987). In the present study, addition of glycerol and sucrose in cryopreservation solution may optimize the freezing injuries during *ex situ* cryopreservation. Therefore, it may be concluded that these two cryopreservation protocols could be applicable for long term *ex situ* conservation of endangered medicinal orchid species of Bangladesh.

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Table 3. Comparative growth index in post-thawed cultured protocorms from cryoprotectant and encapsulation method.

Species	Growth (cm) of protocorms recovered from cryoprotectant method (mean value)	Growth (cm) of protocorms recovered from encapsulation method (mean value)	Growth (cm) of protocorms increased in encapsulation method over cryoprotectant method
Acampe papillosa	1.60	2.50	0.9 (56 %)
Aerides odorata	2.20	3.00	0.8 (36%)
Bulbophyllum lilacinum	2.00	2.40	0.4 (20%)
Cymbidium aloifolium	2.10	2.60	0.4 (19%)
Pholidota pallida	2.00	2.50	0.5 (25%)

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