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RESEARCH ARTICLE

Seed Cryopreservation of Orchid *Coelogyne nitida* (Wall. ex Don) Lindl. Using Air Desiccation and Vitrification Techniques

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Orchid seeds are short-lived and difficult to conserve using conventional methods. Diverse germplasm of orchids occurring in North-eastern parts of India need attention for their long-term conservation. In this study, seed cryopreservation in *Coelogyne nitida* (Wall. ex Don) Lindl. was attempted. A modified TTC test was standardised as a quick estimate of seed viability. *In vitro* asymbiotic seed germination was standardised for determining seed viability for fresh, desiccated and frozen seeds. MS medium supplemented with 0.6 mg l^{-1} GA_3 + 0.8 mg l^{-1} BAP was optimal. Cryopreservation by air-desiccation and vitrification methods were applied with success achieved in both, however vitrification method yielded much higher recovery (83.4%) after liquid nitrogen exposure than the air desiccation (43.5%). Exposure time to PVS2 of 40 min was optimal amongst the three treatment durations during vitrification. This is the first report on successful cryopreservation of *Coelogyne nitida* seeds.

Key Words: Asymbiotic germination, Cryobanking, Orchid, Protocorms, TTC

Introduction

Orchidaceae is the largest angiosperm family next only to Asteraceae with more than 730 genera and over 26,000 species (Chase *et al*., 2015). All members of Orchidaceae family are listed under the Convention on International Trade in Endangered species of Wild Fauna and Flora (CITES) restricting any trade without permission. Orchids share a large chunk of global floriculture trade and hence are given special attention. Historically they had been a favourite subject of scientific experimentations by Charles Darwin who published books on orchids especially on strategies for fertilisation (Darwin, 1862; 1877). His observations formed the basis for 'Theory of Natural Evolution'. He theorised that orchids and their pollinators had co-evolved. In fact the orchid species *Angraecum sesquipedale* is known as Darwin's orchid. Way back in 19th century, several researchers conducted basic studies on orchids (Gosse, 1863; Moggridge; 1865) and their importance for conservation is still increasingly emphasised (Fay, 2018). Orchids are accepted to be difficult to handle (hence termed non-orthodox) and to store; the seeds have cold sensitivity to freezer storage. Explants like protocorms and protocorm like bodies (PLBs) are desiccation sensitive and there have been very limited studies on pollinia/pollen conservation which is essential for controlled, selected pollinations for hybrid vigour and for conservation of nuclear diversity.

About 186 genera and 1,230 orchid species are found in India which constitute almost 10% of the world's orchid flora. North East India, a mega-biodiversity hotspot, harbours an estimated 151 genera and 876 species of orchids (Medhi *et al*., 2012). Seaton *et al*. (2010) has suggested for *ex situ* conservation of orchids in the warming world. Tissue culture studies and conservation of valuable orchid germplasm have been carried out in various laboratories of the world since long (Seaton and Pritchard, 2008). Gangaprasad *et al*. (1999) attempted restoration of orchids using micropropagation. Cryopreservation is increasingly finding applications for long-term conservation of diverse plant species (Chaudhury and Malik, 2016) and cryoprotocols for different orchids

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are reported (see Vendrame *et al*., 2014, Seaton *et al*., 2015, Popova *et al*., 2016). Cryopreservation in various orchid species has yielded varying degree of success with different desiccation levels (optimal being >14%) and various cryotechniques. The present study was undertaken to develop a cost-effective, long-term conservation strategy for *Coelogyne nitida* (Wall. ex Don) Lindl., a sympodial epiphyte. Investigations were done on handling and transport of fruits and seeds, standardisation of tetrazolium test, asymbiotic *in vitro* seed germination and cryopreservation using air-desiccation and vitrification techniques.

Materials and Methods

Collecting of Material

Coelogyne nitida (Wall. ex Don) Lindl. is a sympodial epiphytic orchid (Nongrum *et al*., 2007), that has pseudobulb, and inflorescence is a raceme which flowers from May to June. It is naturally distributed in Sikkim, West Bengal and Arunachal Pradesh. Mature fruit capsules of *Coelogyne nitida* (IC 617089) were harvested about 12 months after anthesis by the NRC Orchids, Darjeeling, West Bengal from the Institute's greenhouse. Immediately after harvest, the pods of *Coelogyne nitida* were packed in plastic bags and sent to ICAR-NBPGR, New Delhi by speed post air carrier for further studies and cryopreservation, where pods were maintained at 15^{o}C until used in for experimentation (within 15 days).

In vitro asymbiotic germination

Coelogyne nitida capsules were washed with tap water and taken inside laminar flow and sterilized by flaming after dipping in 70% alcohol. This was repeated two times as per the procedure of Nongrun *et al.* (2007). The capsules were then slit open under aseptic conditions and seeds, which were abundant and uncountable being thousands to millions in each of the pods, scooped out carefully. For germination, seeds were distributed in glass culture tubes (15 × 2.5 cm) thinly over the surface of 15 ml of six different Murashige & Skoog (MS) (1962) nutrient media aseptically, viz., basal alone or in combination with various growth regulators namely 0.4 mg l^{-1} BAP, 0.6 mg l^{-1} BAP, 0.1 mg l^{-1} GA_3 + 0.2 mg l^{-1} BAP, 0.6 mg l^{-1} GA_3 + 0.6 mg l^{-1} BAP and 0.6 mg l^{-1} GA_3 + 0.8 mg l^{-1} BAP. Each of the media were supplemented with 3% sucrose, 0.8% agar and pH adjusted to 5.8 prior to autoclaving. Cultures were incubated at 25 $\pm$ 2^{0}C under light intensity of 40 µmol m^{-2} s^{-1} for 16/8 h light/dark cycle. Cultures were observed microscopically at weekly intervals to record growth and development evident earliest by swelling in embryos and appearance of protomeristem. Data on days to initiate germination, protocorm, 1st leaf shoot, root were recorded. Percent seed germination was determined after 60 days of culture and calculated by using the formula: % seed germination = number of germinated seeds/total number of seeds × 100.

Viability test using Tetrazolium

Seed viability was also assessed with a modified Tetrazolium test. Seeds (10 mg) were placed in 1 ml tubes and pre-incubated in a 10% (w/v) sucrose solution. After incubation for 24 h at 25°C, sucrose solution was removed and seeds washed twice with distilled water. Seeds were subsequently distributed in three tubes and individually solutions of 0.1, 0.5 and 1% of 2,3,5-triphenyl tetrazolium chloride (TTC), prepared in distilled water, were added to the seeds and incubated in the dark at 35°C for 24 h. After incubation, seeds with liquid drops of TTC solution were placed on a glass slide and observed under microscope. Red/deep pink seeds were counted as viable, while white seeds were counted as non-viable and percentage viability was calculated. Three replicates each with about 300 seeds per sample were used.

Seed Cryopreservation by Air Desiccation

Freshly extracted seeds were placed on sterile filter paper discs in a laminar flow for 4-5 h. Moisture content (MC) of fresh and desiccated seeds was determined gravimetrically using low constant temperature oven method (ISTA, 1985). Seeds, about 300 in number, were placed in pre-weighed moisture bottles. The samples were weighed again after 17 h drying at 103±2^{o}C. The MC was obtained from 2-4 independent determinations (each with about 300 seeds) and expressed as a mean percentage on fresh weight basis. Seeds were suitably desiccated to 16-18% moisture and transferred to sterile 1 ml polypropylene cryovials. These cryovials were subjected to fast freezing at -196^{o}C by direct plunging in liquid nitrogen (LN) (air desiccation-freezing method). After 24 h cryostorage, seeds were thawed at 37±2 ^{O}C and cultured *in vitro* to study survival and recovery, as done with fresh seeds.

Seed Cryopreservation by Vitrification Technique

Freshly extracted seeds (~300) were transferred to 1.2 ml sterile cryovials and treated with 0.5 ml loading solution (0.4 M sucrose, 2 M glycerol in basal MS medium) for 20 min at 25°C. Loading solution was replaced with 0.5 ml Plant Vitrification Solution 2 [PVS2: 30% (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) dimethyl sulfoxide and 0.4 M sucrose desolved in MS medium (pH 5.8)] (Sakai *et al*., 1990) for 20, 40 and 60 min at 25°C. Cryovials containing seeds in PVS2 solution were plunged rapidly in LN and held for 1 h. Subsequently, the cryovials were thawed in a water bath at 38±2°C for 1 min with vigorous shaking. The PVS2 was immediately replaced with 0.5 ml unloading solution (1.2 M sucrose in MS basal medium) and incubated at 25°C for 20 min. The solution was removed, seeds blotted dry on sterile filter papers and then cultured *in vitro* to study survival and recovery as done with fresh seeds. In all, growth regulators-supplemented, five MS culture media (detailed in Table 2), were used for recovery, two of them with BAP alone in two concentrations namely 0.4 mg l^{-1} and 0.6 mg l^{-1} and with different concentrations of GA_3 (0.1 mg l^{-1} and 0.6 mg l^{-1}). Experiments were repeated two times.

Transfer of Plantlets to Pots

Seedlings ~4–5 cm tall and with well-formed roots, raised from cryo-retrieved samples, were removed from culture vessels and roots washed thoroughly with tap water to remove adhering medium without damage. Ten of the seedlings were then transplanted in plastic pots (10 × 7 cm size) containing mixture of coco peat, vermicompost and perlite in 1:1:1 ratio. Plantlets were covered with a pierced transparent polythene bag, and allowed to grow in the laboratory under ambient conditions (at 24°C and 80% RH) for 3 weeks with regular watering. Later, pots were shifted outside the lab in partial shade where minimum and maximum temperatures were 18 and 25°C, respectively, and RH was 70–80 %. Plantlets were watered on alternate days and fed with 1/10 strength MS nutrient salt solutions every two weeks. Establishment of plants was assessed after 90 days.

Data Analysis

Data generated in *in vitro* asymbiotic seed germination and cryopreservation experiments were statistically analysed using CRD by Panse and Sukhatme (1985). SPSS software was used to generate ANOVA analyses at the 0.1% level of probability. In the Tables, values followed by the same letter in a column are not significantly different using DMRT.

Results

Healthy undamaged mature pods of *Coelogyne nitida* were successfully transported after harvesting from North East India to ICAR-NBPGR, New Delhi, a distance of about 1500 km. The average weight of pods was 1.71 g and average size was 6.03 cm in length and 1.03 cm in breadth (Fig 1a). The colour of extracted seeds (Fig 1b) ranged from white, yellow and green in different pods. Immature seeds were invariably white and pale. About 15% of seeds from the total pods received were empty, as they lacked the embryos. The MC of fresh seeds averaged 45.9%.

Using TTC test, seed viability of 18.0%, 46.2% and 82.5% was recorded for fresh seeds incubated in 0.1% (Fig 1c), 0.5% (Fig 1d) and 1% (Fig 1e) solutions. The values achieved by 1% TTC solution test for viability were similar to that achieved by *in vitro* germination (Table 1). Seeds after cryoexposure were also randomly checked for seed quality using 1% TTC solution and viability values were similar to those achieved by *in vitro* germination (data not presented here).

Under *in vitro* conditions, fresh seeds cultured on MS basal medium exhibited swelling by 28 days, protocorms (Fig 1b) appeared by 40 days, shoots (Fig 1d) and roots initiated by 71 and 101 days, respectively (Table 1). Amongst the five growth regulators supplemented MS medium, best response was observed with that supplemented with 0.6 mg l^{-1} GA_3 and 0.8 mg l^{-1} BAP and hence data has been presented for this medium in present studies on asymbiotic seed germination. In comparison to MS basal, the responses were faster by 2-5 days for seeds inoculated on MS medium supplemented with 0.6 mg l^{-1} GA_3 and 0.8 mg l^{-1} BAP. Mean seed germination recorded after 60 days of culture was 82%. Seeds desiccated to 17.93% MC and those desiccated and exposed to LN were similarly tested for asymbiotic germinaton on these two culture media namely MS basal and MS with 0.6 mg l^{-1} GA_3 and 0.8 mg l^{-1} BAP. For desiccated unfrozen seeds, initiation of seed germination was observed, within 20 days, on medium supplemented with growth regulators whereas in MS basal media, it was by 29th day of culture. Protocorms were visible by 42 days on MS basal and few days earlier (by 38 days) on growth regulators-supplemented media. Healthy upright shoots emerged by about 80 days in both the culture

media (Fig 1e). In next 30-32 days, rooting was initiated on both the media. Final seed germination percentage was 45.32% on MS basal medium and 75.5% on growth regulators-supplemented MS media.

Desiccated frozen seeds were thawed and germinability assessed on two media used for fresh seeds (Table 1). Initiation of seed germination was observed earlier, within 25 days, on growth regulator-supplemented medium, whereas, on MS basal medium, it was slower and observed by 31st day of culture which was a delay of 3-5 days than in their respective nonfrozen controls. Protocorms initiation and shoot formation was also delayed by 4-8 days in comparison to their respective nonfrozen controls. Rooting in frozen seeds was initiated on MS basal medium by 128 days, a delay of 18 days compared to unfrozen seeds and on growth regulator-supplemented media by 124 days, a delay of 6 days compared to nonfrozen seeds. After 18 weeks of culture, germination was recorded as 16.36% on MS basal medium and 43.5% on MS media supplemented with growth regulators. Hence, lower germinability values were observed in LN exposed seeds when compared to fresh and nonfrozen seeds. In all cases, however, growth regulator-supplemented media were better for germinability. The plantlets raised from the two media appeared normal and healthy and could be transferred to pots with 100% success for one month in healthy conditions (Fig 1f).

Vitrification method was attempted with freshly extracted seeds to investigate the role of cryoprotectants in improving survivability of seeds after LN exposure. Seeds subjected to freezing preceded by 20 min PVS2 showed germination ranging from 80% on MS medium supplemented with 0.6mg l^{-1} GA_3 + 0.8mg l^{-1} BAP to 55% on MS medium supplemented with 0.1 mg l^{-1} GA3 + 0.2 mg l^{-1} BAP (Table 2). Seeds subjected to freezing preceded by 60 min PVS2 treatment showed germination ranging from 70.5% on MS medium supplemented with 0.6 mg l^{-1} GA_3 + 0.8mg l^{-1} BAP to 55.5% on MS medium supplemented with 0.6 mg l^{-1} BAP. Highest recovery values were recorded for seeds subjected to freezing preceded by 40 min PVS2 treatment. Germination as high as 79 to 83% was recorded on MS media supplemented with different combinations of GA_3 + BAP and 75% on MS media supplemented with BAP alone. Overall MS medium supplemented with 0.6 mg l^{-1} GA_3 + 0.8 mg l^{-1} BAP was optimal for all the treatments.

Discussion

Orchids are priority to be conserved through biotechnological interventions (Pritchard, 2016). Generally seeds are the preferred propagules for propagation and storage. However, orchid seeds are reportedly difficult to handle due to varying proportions of seeds formed without embryos and inherently low germination rates. Responses due to heat and cold sensitivity are other causes misunderstood as mishandling (Pritchard, 1989). Despite these difficulties, orchid seeds with low MC are the much preferred explants compared to protocorms, which suffer cryoinjury due to their inherent high moisture levels. Although several reports exist on success in orchid seed storage in conventional -20^{o}C genebanks (Seaton *et al*., 2010), cryobanking has shown greater potential as the viable method (Merritt *et al*., 2014; Popova *et al*., 2016). Generally laboratories worldwide

Table 1. Asymbiotic *in vitro* seed germination and cryopreservation by desiccation, in *Coelogyne nitida* using 300 seeds in 3 replicates

LN exposure	Media Composition	Days taken for initiation of				Final germination Percentage %
		Seed swelling	Protocorm development	Shoots appearance	Roots appearance	
Fresh Seeds	MS Basal	28	40	71	101	81.5±5.7^{ab}
	MS+0.6mg l^{-1} GA_3+0.8mg l^{-1} BAP	26	36	66	97	82.5±1.1^{a}
Desiccated non-frozen controls	MS Basal	29	42	79	110	45.3±8.9^{d}
	MS+0.6mg l^{-1} GA_3+0.8mg l^{-1} BAP	20	38	81	118	75.5±5.6^{c}
Desiccated frozen	MS Basal	31	47	81	128	16.3±4.1^{e}
	MS+0.6mg l^{-1} GA_3+0.8mg l^{-1} BAP	25	46	85	124	43.5±0.8^{d}

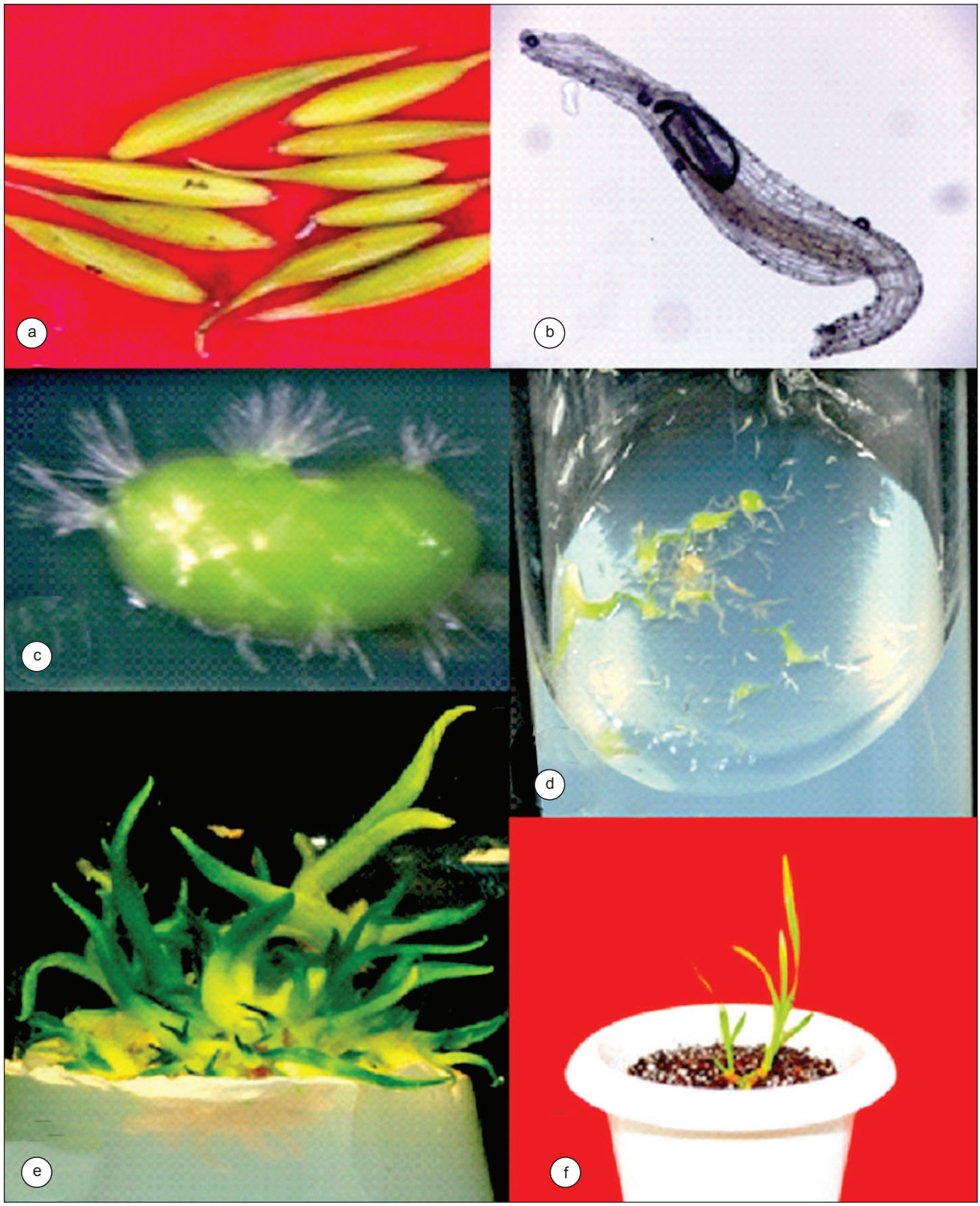

Fig. 1. Asymbiotic *in vitro* seed germination in *Coelogyne nitida*
a. Freshly collected pods; b. Single seed showing well defined embryo; c. *In vitro* A raised protocorm with rhizoids; d. First shoot formations from protocorms; e. Well developed shoots showing root initiation; f. Seedling transferred to pot

do not have the necessity to transport material from one lab to another but use the germplasm locally. This is the first effort to collaborate with Institute situated as far as 1500 km away but needed to exchange *Coelogyne nitida* germplasm in viable condition and fresh pods received through air travel showed reasonably good seed viability, indicating no transportation losses.

Germination tests are the ideal method for correct evaluation of seed quality. However, time taken to evaluate orchid seeds for their full germination potential is several weeks (Merritt *et al.*, 2014). This necessitates use of vital stain 2,3,5-triphenyl tetrazolium chloride for rapid estimation of viability, which is a widely acceptable test for orchid seeds. In the present study, viability determination using 1% TTC preceded by sucrose incubation gave a fair estimate of viability comparable to *in vitro* germination. Hosomi *et al.* (2011) earlier reported an improved colouration and distinction between dead and live seeds of *Cattleya* in TTC test following the pre-conditioning with sucrose. Orchid seeds, due to minute size and oil reserves, often do not have their metabolism activated (Arditti, 1980) and depend on external sucrose for energy source which may be the case for optimal results in TTC test in present studies, using sucrose incubation overnight. For orchid species possessing dark or impermeable seed coats, an improved TTC testing has also been devised that includes a bleaching step (Custodio *et al*., 2016).

For mass propagation of selected orchid species, asymbiotic *in vitro* seed germination and *in vitro* establishment have been standardised by various workers (Arditti, 1984; Popova *et al*., 2016). This helps in conservation of declining orchid population in nature. Role of different culture media for asymbiotic seed germination has been investigated for several orchid species. In our studies, Murashige and Skoog (MS) medium supplemented with GA_3 and BAP was suitable for obtaining survival after various treatments, since fresh seeds exhibited high germinability (82%). This germination value was better than that reported earlier by Nongrum *et al*. (2007) where four culture media viz. Knudson C, Vacin and Went, Mitra and MS were used. MS was also one of the suitable media for further growth of protocorms and plantlets, in their studies. In present work, the microscopic examination showed 10-15% seeds without any embryos corroborating the results of viability achieved with *in vitro* germination and TTC method. Growth regulators reportedly do not have a major role in orchid seed germination, but are essentially needed for subsequent protocorm and seedling formation. Further, long-term conservation of orchid seeds for assured high viability retention for infinite periods needs to be devised for uninvestigated species. Cryopreservation has been increasingly employed with different orchid species with high predictable success in seeds, for both epiphytic and terrestrial species, and for pollen/ pollinia (reviewed by Popova *et al*., 2016). In this context, present study on low temperature tolerance of seeds of *Coloegyne nitida* was carried out using two cryopreservation protocols. There are no reports so far on long-term cryopreservation of seeds of *Coelogyne nitida*.

At ultra-low temperature storage, role of optimal moisture window, achieved by partial drying, for best results in terms of high viability with normal protocorm development has been emphasised (see Popova *et al*, 2016). Most conventional method is to desiccate seeds between 13 to 17% moisture followed by direct plunging in LN. In exceptional cases, seeds with high moisture between 24-25% have been reported to also survive direct LN freezing (Nikishina *et al*., 2001; Wu *et al*., 2013). In the present study, a safe moisture level of 16-18%, reported for most of the orchid species was employed before freezing seeds to avoid any desiccation and also freezing injury. Desiccation of seeds resulted in decline in germinability which was substantial (37% decline) using MS basal medium. Growth regulator supplementation,

Table 2. Vitrification freezing of seeds of orchid species *Coelogyne nitida* using 300 seeds in 3 replicates

Media Composition	% Germination after PVS2 exposure of (min)		
	20 min	40 min	60 min
MS+0.4 mg l^{-1} BAP	67.8±4.0^{c}	75±2.8^{d}	60.5±2.8^{c}
MS+0.6 mg l^{-1} BAP	65.5±5.7^{c}	75±5.7^{d}	55.5±1.4^{d}
MS + 0.1 mg l^{-1} GA_3 + 0.2 mg l^{-1} BAP	55.0±2.3^{d}	78.5±1.1^{cd}	65.7±2.8^{b}
MS + 0.6 mg l^{-1} GA_3 + 0.8 mg l^{-1} BAP	80±3.2^{a}	82.2±1.4^{ab}	70.5±1.4^{a}
MS + 0.6 mg l^{-1} GA_3 + 0.6 mg l^{-1} BAP	71.4±4.9^{b}	83.4±1.7^{a}	59.7±1.4^{d}

however, showed improved response (only 6% decline in germination) for desiccated seeds. In addition, various initiation responses of seeds germination and seedling growth were slower after seed desiccation (Table 1). Seeds after freezing showed a further decline in germinability indicating freezing injury. However, recovery percentage was up to 43% while using growth regulator-supplemented media. Growth regulators were, thus important for optimising recovery of desiccated and cryo-exposed seeds.

Vitrification is a freezing technique which chiefly comprises seven steps namely preculture, osmoprotection, dehydration with highly viscous vitrification solutions like PVS2 (Sakai and Engelmann, 2007), rapid cooling, rapid warming, dilution/exclusion of vitrification solution from the explant and subsequent recovery growth. Vitrification solution is attributed with three functions (Volk and Walters, 2006) i.e. replacing cellular water, altering the freezing behaviour of the cells and impeding water loss on drying. Carefully selected exposure duration to these solutions is the key to success as they themselves can cause stress of osmotic injury and dehydration. PVS2 has proven successful with several orchid species with immature and mature seeds (Hirano *et al*., 2011). Successful reports exist on vitrification of orchid seeds both with or without pre-culture in high sucrose before subjecting to cryopreservation (Merritt *et al*., 2014). In present study, high seed survival (83.4%) was achieved following vitrification (without any preculture treatment) that was as good as in nonfrozen controls (82.5%). Exposure time of 40 min to PVS2 was optimal amongst the three treatment durations and MS media with different concentrations and combinations of GA_3 and BAP were most suitable for recovery. Exposure duration of 20 min was next best followed by 60 min exposure. During vitrification, dehydration of seeds could be achieved using high viscosity cryoprotectants which reportedly leads to glass formation omitting any ice crystallisation (Sakai and Engelmann, 2007). Healthy plantlets were recovered after vitrification defining a successful cryopreservation methodology. Hence, two long-term, economical and safe conservation methods of *Coelogyne nitida* seeds using air desiccation-freezing and vitrification were developed. Lately, cryopreservation has been emphasised as the most suitable alternative bioresources germplasm storage of seeds of short-lived orchid species that cannot be seed-banked at conventional temperatures of -20^0C effectively (Streczynski *et al.*, 2019). Based on this study attempts are being made to cryobank germplasm of *C. nitida* at ICAR-NBPGR, New Delhi, in the form of seeds.

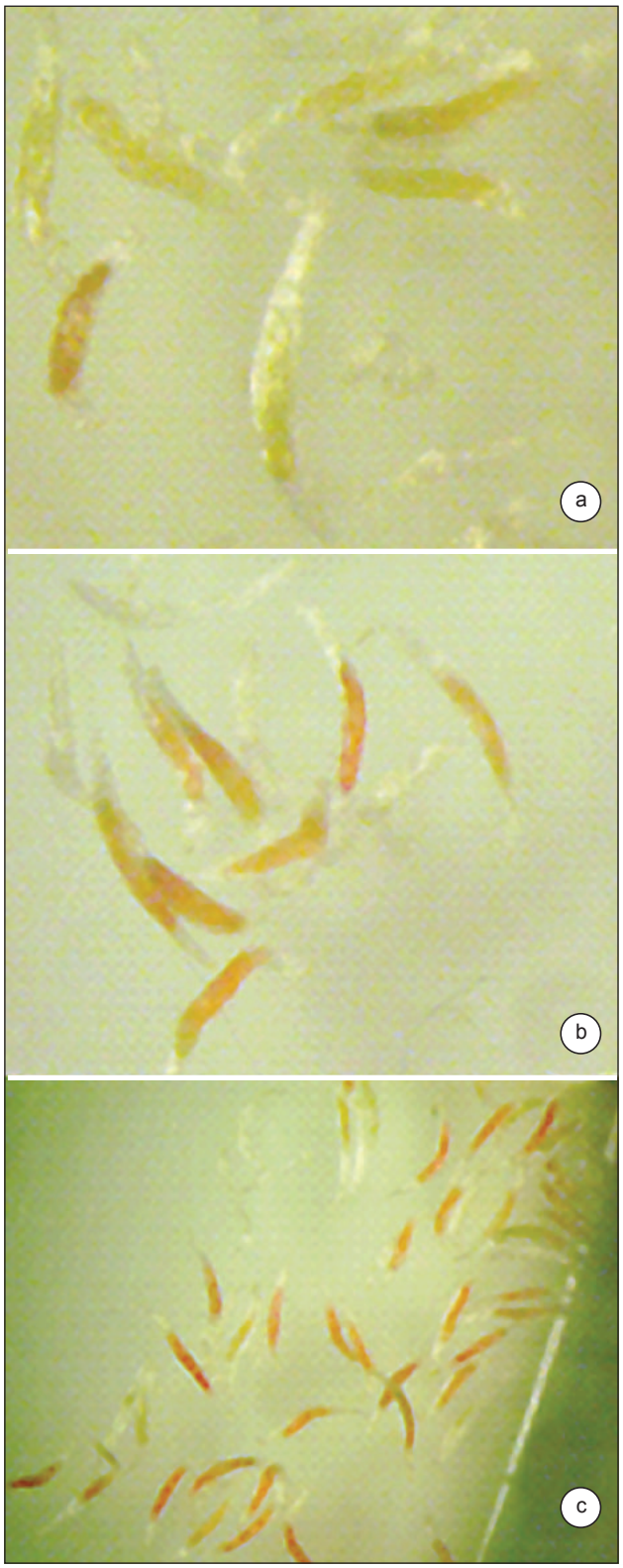

Fig. 2. *Coelogyne nitida* fresh seeds tested for viability using different concentrations of TTC solution
a. 0.1% TTC solution; b. 0.5% TTC solution
c. 1% TTC solution

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