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Asymbiotic germination and seed storage of *Paphiopedilum insigne*, an endangered lady's slipper orchid

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ABSTRACT

The present scenario of urbanization and commercialization has adversely affected orchid's population; as a consequence they are diminishing from the nature very rapidly. *Paphiopedilum insigne* (lady's slipper orchid) is one such orchid of horticultural importance. Apart from being listed as endangered in IUCN red data list; it finds a place in Appendix I of CITES in the global platform. In the present study, capsules <180 days after pollination (DAP) were found to be immature and 180 DAP was found optimal for seed germination. Capsules >240 DAP were found to have mature seeds that were treated prior to germination and TTC tested for viability. Seeds derived from 180 DAP capsules showed the highest germination of 88.5% in modified Burgeff medium (BG1) with initiation in 26 days. Incorporation of plant growth regulators like 5 μM kinetin (KN) + 10 μM indole-3-acetic acid (IAA) in 1/2 MS medium influenced the stage-wise development of the seedlings in a short duration. Mature seeds stored at −196 °C for 360 days followed by pre-treatment with 3% NaOCl showed viability of 70.5% and recorded germination of 79.2%. Sixty-nine percent of the plantlets were successfully hardened and acclimatized in the green house.

1. Introduction

There is an unmatched fascination that we the people have with orchids and their unique beautiful flowers. However, there is an undying fact that their very existences in nature today, is deteriorating globally. *Paphiopedilum insigne* (known as lady slippers), is one such beautiful terrestrial orchid. It is distributed in small pockets of Northeast India (namely, Nagaland, Manipur, Mizoram and Meghalaya which is part of the Indo-Burma mega biodiversity hotspot of India) and other Southeast Asian countries like China; Myanmar; Thailand; Laos and Vietnam (Chowdhery, 2004; Tandon and Kumaria, 2011; http://www.iucnredlist.org/). This species is marketed as an attractive potted plant due to long shelf-life (60–90 days) of the flowers which are used as parents in producing hybrids in breeding programmes. There are a few reports on ethno-botanical utilization of *P. insigne* as a medicine in curing stomach ailments (Friesen and Friesen, 2012). Poor germination and slow plantlet growth have added to it being rare in the wild (Yam and Arditti, 2009; Zeng et al., 2015). In the present scenario, the orchids are experiencing a steady decline from their natural habitats. With concerns on prioritizing for conservation, *P. insigne* has not only been included under the latest IUCN red list as endangered plant (Rankou and Kumar, 2015; http://www.iucnredlist.org/) but, also enlisted in Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES).

Although orchid seeds are the best source to conserve their genetic diversity still, they are under-rated, hence rarely collected, sowed or stored. The heterogeneous nature of seeds makes them suitable for conserving genetic diversity of plant populations in nature. Orchid seeds too exhibit this nature in conjunction with other features like minute seed size and ample availability per capsule. Despite of several attempts on preserving orchid population in the wild yet, they are still abating at an alarming rate. There is a need to strategize *in-situ* and *ex-situ* conservation methods to help preserve this orchid. Ideally, approach of conservation is *in-situ* (Tandon, 2004), however this practice is unsafe due to many anthropogenic activities. Alternatively, *ex-situ* conservation offers not only safer security backup system for conservation (Chugh et al., 2009; Engelmann, 2010) but also allows accessibility for research work evaluation. *In-vitro* technologies like asymbiotic seed germination, mass propagation, short-long term storage *etc.* based on *ex-situ* conservation have been used to conserve rare, endangered and threatened plants especially orchids (Arditti, 1994; Kumaria and Tandon, 2001; Decruse et al., 2003; Dutra et al., 2008; Yam and Arditti, 2009; Mohanty et al., 2012; Bhattacharyya et al., 2017). Long-term preservation of orthodox seeds *via.* cryopreservation in liquid nitrogen (LN; −196 °C) helps in maintaining the plant germplasm in stasis for years and is also applied for rescue of rare and endangered plant species (Engelmann,

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2004; Nikishina et al., 2007; Thammasiri and Soamkul, 2007; Hirano et al., 2009; Kaczmarczyk et al., 2011). Terrestrial orchids unlike their epiphytic counterparts, are difficult to germinate *in-vitro* and fail to establish in soil on a large scale (Batty et al., 2001; Stewart and Kane, 2006; Swarts and Dixon, 2009). There are a number of reports on asymbiotic germination of immature seeds of this genus which varies from species to species (Arditti and Ernst, 1984; Kauth et al., 2006; Zeng et al., 2013). Mature orchid seeds may have a greater potential for propagation and storage because of fully developed testa and lower water content (Miyoshi and Mii, 1998; Fu et al., 2016). Yet, for many species, prospects for this kind of conservation are hampered by poor storage conditions and regeneration protocols need to be standardized (Stimart and Ascher, 1981; Long et al., 2010; Merritt et al., 2014; Zeng et al., 2015).

Therefore, in this present study, we report an effective protocol for *ex-situ* conservation of *P insigne* with high frequency seed germination, storage of mature seeds and seedling growth comparatively in a shorter period.

2. Materials and methods

2.1. Capsules collection and sterilization

The plants of *P. insigne* were collected from the nurseries of Upper Shillong, Cherrapunjee, Mawsynram and maintained in the greenhouse of Plant Biotechnology Laboratory, Department of Botany, North-Eastern Hill University, Shillong, India. The collection is in compliance with the fulfilment of legal requirements. During the flowering season 70 flowers were hand pollinated by dusting pollens onto the stigma of the same flower and covered with polythene bags. From the time of hand pollination, the immature capsules and mature seeds were harvested at interval of 30 days. The capsules <240 days after pollination (DAP) were washed thoroughly with tween 20 and rinsed under running tap water 3–4 times. These capsules were then treated with 70% alcohol for 30s followed by flame sterilization. Mature seeds >240 DAP were collected from the polythene bags and subjected to various pre-treatments like pre-soaking in water for 30 min (control), 1%, 3%, 5% and 7% of NaOCl (4% available chlorine; Himedia) for 30 min, After every pre-treatment with NaOCl, the seeds were rinsed with sterile distilled water 6–8 times.

2.2. Asymbiotic seed germination of immature and mature seeds

Immature seeds were scooped out of the capsule and inoculated on different media namely, Murashige and Skoog (MS), BM Van Waes (BM), modified Burgeff (BG1), 1/2 MS (Table 1) contained in 25 × 150 mm glass test tubes each containing 10 ml of medium. The cultures were incubated in the dark for 10 days, and to16 h photoperiod at 50 μmol m^2 s^{-1} light intensity. Initiation of seed germination was observed after every 10 days but percentage germination was recorded 60 days after inoculation (DAI). Ten replicates were maintained and the experiment was repeated thrice.

Similarly, pre-treated mature seeds were inoculated for seed germination while the seed morphology for mature seeds after every treatment was observed under Scanning Electron Microscopy (SEM). For SEM studies, seed samples were air dried in laminar air flow for 1 h followed by drying with a JFD-310 freeze dryer and affixed to aluminium stubs and coated with gold in a JFC-1100 (JEOL) ion sputter coater and observed using a JEOL, JSM-6360 SEM at 20 kV (SAIF, NEHU, India).

The proliferation of protocorms into seedlings at different developmental stages of protocorms was assessed (Table 2). Thirty days old protocorms were subjected to different concentrations of plant growth regulators namely, 6-benzylaminopurine (BAP), kinetin (KN), α-naphthalene-acetic acid(NAA) and indole-3-acetic acid (IAA) ranging from 0 to 25 μM concentrations singly and in combination, were incorporated in the optimal medium for seedling growth assessment. Percentage response of protocorms at each stage and cumulative developmental stages of the protocorms/seedlings were recorded after 30 DAI. Growth parameters like shoot number and length and root number and length were further evaluated separately. The data were recorded at 30 DAI. Twenty protocorms were taken for each treatment and all experiments were repeated thrice.

Table 1
Composition of organic and inorganic nutrients used in various media.

Constituents	MS	1/2 MS	BG1	BM
Inorganic nutrients mg/ml)				
NH_4NO_3	1650	825	–	–
KNO_3	1900	950	–	–
KH_2PO_3	170	85	–	–
$Ca(NO_3)_2 \cdot 4H_2O$	–	–	1000	–
$MgSO_4 \cdot 7H_2O$	370	185	250	100
$(NH_4)_2SO_4$	–	–	250	–
$MgCl_2 \cdot 6H_2O$	–	–	–	–
KCL	–	–	250	–
$KH_2PO_4 \cdot 3H_2O$	–	–	250	300
KI	0.83	0.41	–	80
H_3BO_3	6.2	3.1	–	6.2
$MnSO_4 \cdot 4H_2O$	22.3	11.15	–	100
$ZnSO_4 \cdot 7H_2O$	0.25	0.12	–	10
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.12	–	0.25
$CuSO_4 \cdot 5H_2O$	0.25	0.12	–	0.025
$CoCl_2 \cdot 6H_2O$	0.025	0.012	–	0.025
$FeSO_4 \cdot 7H_2O$	27.8	13.9	20	27.3
$Na_2EDTA \cdot 2H_2O$	37.3	18.65	–	37.3
$CaCl_2 \cdot 2H_2O$	440	220	–	–
$CO(NO_3)_2 \cdot 6H_2O$	–	0.05	–	–
$NaH_2PO_4 \cdot 2H_2O$	–	250	–	–
$MnCl_2$	–	–	–	3.9
H_3BO_3				10
Organic nutrients (mg/ml)				
Thiamine HCl	0.1	0.1	–	0.5
Nicotinic acid	0.5	0.5	–	5
Pyridoxine HCl	0.5	0.5	–	0.5
Glycine	2	2	–	2
Citric acid	–	–	90	–
Biotin	–	–	–	0.05
L glutamine	–	–	–	2
Casein hydrolysate	–	–	–	500
Meso Inositol	100	100	–	–
Glucose	–	–	20,000	
Sucrose	30,000	30,000	–	20,000
Activated charcoal	2000	2000	2000	2000
Agar	8	8	11	12
pH	5.8	5.8	5.3	5.2

2.3. Mature seed storage studies

Approximately, 200 mg mature seeds (>240 DAP) were placed per 2-ml sterile cryovials (polypropylene, Tarsons Pvt. Ltd., India.) followed by fixation in cryocane (Tarsons Pvt. Ltd., India) and stored at different temperatures for 280 days. After every 30 days of storage, the seeds were sterilized according to the protocol standardized for mature seeds and inoculated on the optimized regeneration medium. In case of seeds stored directly in LN, thawing was done by dipping the cryovials in distilled water at 45 °C for 2 min followed by the same

Table 2
Developmental stages of protocorm to seedlings.
(Modified from Stewart et al., 2003)

Stages	Description
I	Embryo enlarged, testa ruptured (=Germination)
II	Appearance of protomeristem
III	Emergence of two-first leaf primordia & elongation of shoot
IV	Root development