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In vitro seed storage of *Paphiopedilum villosum* Lind., an endangered lady's slipper orchid

*Reema Vareen Diengdoh, Suman Kumaria and Meera Chettri Das**
Plant Biotechnology Laboratory,
Department of Botany,
Centre for Advanced Studies,
North-Eastern Hill University, Shillong-793022, Meghalaya, India,
*Corresponding author: dasmeera73@gmail.com

Abstract

Urbanization and commercialization has adversely affected orchid's population. As a result, they are diminishing from the nature very rapidly. Terrestrial orchid, Paphiopedilum villosum (lady's slipper orchid) is one such with 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 vitro storage with complete regeneration protocol plays an important role for conservation of endangered species. Seed storage is the best option for conservation, however this can be accomplished only after standardizing the correct age of the seed, different temperatures for storage, optimal media for regeneration, etc. There are number of reports suggesting the feasibility of immature orchid seeds for storage. However, storage studies on mature dehisced orchid seeds are few. Therefore, in the present study, we attempted to standardize a protocol for storage of mature dehisced seeds of P. villosum at three different temperatures (0˚C, 25˚C and -196˚C) followed by post storage germination and regeneration studies. We report the feasibility of storing P.villosum seeds in LN (-196˚C), BG1 as the optimum germination medium for the seeds and MS medium for conversion into seedlings. MS medium incorporated with 20 µM BAP+5 µM IAA proved better for growth and development of the seedlings and hence can be used for multiple subcultures till the seedlings are ready to transfer.

Keywords: Endangered orchids, Mature seeds, Liquid Nitrogen, Storage studies

Introduction

The heterogeneous nature of seeds makes 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. 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.*, 2001comprising orchid (Caladenia arenicola; Swarts and Dixon, 2009; Zeng *et al.,* 2013). Besides other factors, prerequisite determination of correct age of the capsule is necessary for maximum *in vitro* germination of orchid seeds. Practically immature undehised capsules age of 4-6 months old are reported to have

high *in vitro* germination rate (Balilashaki *et al.,* 2015; Zhang *et al.,* 2013). On contrary, mature seeds of older capsules results in lower seed germination, due to dormancy owing to many factors of which lignification and cutinisation plays an important role (Yamazaki and Miyoshi, 2006). At the same time, mature orchid seeds may have a greater potential for propagation and storage as orchids have fully developed testa and lower water content and dormancy can be broken (Miyoshi and Mii, 1998; Hu *et al.,* 2013; Fu *et al.,* 2016).

Temperature plays an important role for seed storage. Different temperatures are reported to have potential for prolonged seed storage in different species. Ideally, seed storage in Liquid Nitrogen (-196^{0}C) is an efficient *ex situ* strategy to safeguard the species, but unfortunately, orchid seeds conservation is hampered by poor storage conditions and regeneration protocols that needs to be standardized (Long *et al.,* 2010; Merritt *et al.,* 2014a; Zeng *et al.,* 2015). The advantages of liquid nitrogen storage are: storage for an indefinite period, genetic stability of the individuals, reduced infrastructure, can have independent energy and the stored genetic material does not require manipulation (Cerna *et al.,* 2018).

Therefore, we attempted to store *Paphiopedilum villosum* mature seeds at different temperatures followed by viability test as well as *in vitro* germination after the storage to confirm its feasibility for *ex situ* conservation. *Ex situ* conservation offers not only safer security backup system for conservation but also allows accessibility for research work evaluation (Chugh *et al.,* 2009).

Materials and methods

Seed collection: Dehisced seeds of *P. villosum* were collected from desiccated and dehisced capsules in the polythene bags after 240 days of pollination (DAP). Approximately, 200 mg mature seeds were placed per 2 ml sterile cryovials (polypropylene, Tarsons Pvt. Ltd. Kolkata, India.) followed by fixation in cryocane (Tarsons Pvt. Ltd., Kolkata, India).

Storage of seeds: These cryovials were labelled and stored at different temperatures namely, 0˚C, 25˚C and LN (-196˚C). All seeds were stored for a total of 360 days at different temperature conditions.

Sterilization: Under sterile conditions, after every 30 days of storage, one vial each of stored seeds in 0˚C, 25˚C were sterilized with 3% NaOCl for 30 min followed by rinsing in sterile water. For the seeds stored in -196 ˚C, rewarming was done by dipping the cryovials in distilled water at 45°C for 2 min followed by the same procedure for sterilization.

Viability testing and *in vitro* germination: Simultaneously, after every storage interval, seeds from each storage conditions were divided into two equal proportional quantities. One part was subjected to viability test using 1%TTC (Vujanovic *et al.,* 2000) where, the seeds were soaked 24 h in 1% TTC solution. This was followed by slide observation where seeds showing some degree of pink or red colour were considered viable and scored

accordingly under the microscope. For each treatment, 6 replicates were maintained and the experiment was repeated thrice. The other part of the stored seeds after sterilization were inoculated on different media BG1, ½ MS (Murashige and Skoog), MS and BM terrestrial medium (BM) to assess the optimized growth and development of the seedlings at different stages.

For recording the 1% TTC viability test, the seeds were randomly removed and dispersed in a drop of water on a glass slide and observed under a light microscope. The percentage of seed germination was calculated using the formula:

$$\text{Viability \%} = \frac{\text{No. of pink or red seeds considered viable}}{\text{Total no. of seeds observed}} \text{ x } 100$$

Seeds were considered to have germinated upon emergence of the embryo from the testa (Kumaria and Tandon, 1991). Germination percentage was recorded 60 days after inoculation (DAI). For recording the germination percentage, the seeds were randomly removed and dispersed in a drop of water on a glass slide and observed under a light microscope.

The percentage of seed germination was calculated using the formula:

$$\text{Germination \%} = \frac{\text{No. of seeds showing emergences of the embryo from testa}}{\text{Total no. of seeds observed}}$$

Culture conditions and Statistical analysis: pH of the medium was adjusted to 5.8 using 1N NaOH prior to autoclaving at 15 psi, 121ºC for 15 min. All the cultures were incubated at 25±2ºC and 16 h photoperiod at 50 µM $m^{-2}s^{-1}$ light intensity. Ten replicates were maintained and the experiment was repeated thrice. Statistical analysis was done by analysis of variance (ANOVA) at $p \geq 0.05$ and means compared with Turkey's test using one-way ANOVA (PC version) Origin 8.0 NORTHAMPTON.

Results and discussion

Germination percentage ranging from 78.9 ± 2.6 - 81.5 ± 1.5 was recorded in mature seeds of *P. villosum* stored at -196 °C with similar viability percentage i.e., 78.7 ± 2.4% - 80.1 ± 1.9%, (pink colouration) over 360 days' storage (Figure 3b). The results showed consistency without any significant variation in both germination and viability percentage of mature seeds stored at -196 °C irrespective of different storage time suggesting the feasibility of mature seed storage upto and beyond 360 days. Immature seeds have the zygotic embryo which is not fully developed and the testa might not be lignified, allowing it to be permeable to water and nutrient. But in case of mature seeds, a fully formed testa and low water content may show superior potential for storage as reported earlier (Miyoshi and Mii, 1998; Zhang *et al.,* 2015; Fu *et al.,* 2016). Seeds or shoot tips have been successfully stored in liquid nitrogen (LN) providing protection with the ability for revival and plant regeneration when needed in future. Cryopreservation or LN storage has been used for seed and shoot-tip conservation, providing long-term storage (Engelmann, 2004; Li and Pritchard, 2009; Pritchard and Nadarajan, 2009; Reed

et al., 2011). Poor response was observed in seeds stored at 25 °C, both using TTC test as well as *in vitro* germination suggesting that at higher temperature the seeds are subjected to excessive dehydration stress leading to imbibition injury inducing rapid rehydration in free water as reported by Hirano *et al.* (2011).) Depending on the duration method and temperature adopted, drying and long-term storage may lead to considerable reduction in germination or to eventual death of the seeds. Storage of orchid seeds at higher temperature and humidity can result in reduced seed vigour, low germination and reduced seedling survival (Bewley and Black 1994; Begnami and Cortelazzo 1996). These conditions are believed to affect protein metabolism (Bewley and Black, 1994) and cause a reduction in seed biochemical activity (Bailly *et al.,* 1996; Begnami and Cortelazzo, 1996). Whereas except for 30 days storage at 25°C, there is significant difference in both germination and viability percentage of mature seeds of *P. villosum* stored at both 25°C and 0°C favouring the method of *in vitro* germination to viability test as post storage survival. In orchid seeds, the TTC test has been successfully used for estimation of survival rate (Singh 1981; Van Waes and Debergh 1986a,b) and shown to correlate well with germination percentages after long-term storage (Shoushtari *et al.,* 1994). In contrast, TTC staining could result in overestimation of seed viability when seeds were subjected to prolonged exposure to sodium hypochlorite, which caused high TTC stainability of the embryo because of release of dehydrogenase from damaged embryos (Lauzer *et al.*, 1994).

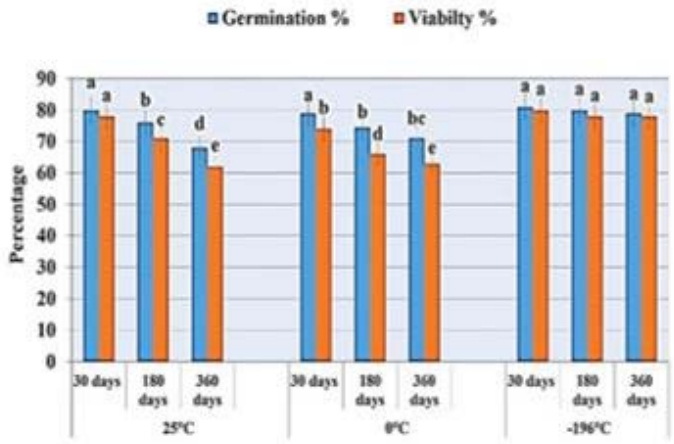


Figure 1: Effect of storage of exposed seed (>240 DAP) on germination recorded after 30 DAI on BG1 medium and 1% TTC viability test of *P. villosum*. Bars with different letters signify statistically different means according to Tukey's test ($p \geq 0.05$)

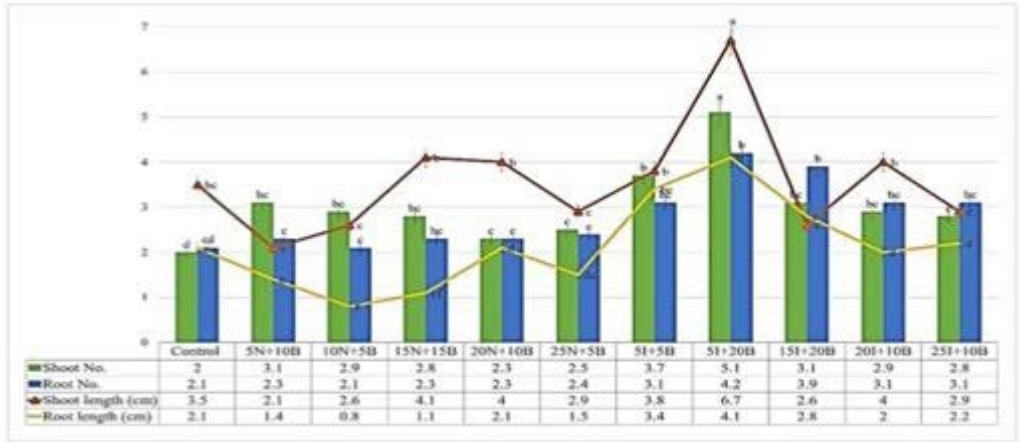
In the present study, reduction in germination as well as viability percentage of the mature seeds in both the temperatures was recorded with increase in storage time (Figure 1).

Table 1: Effect of media on different developmental stages of protocorms (% conversion) of *P. villosum* recorded after 30 DAI post storage in LN.

Media	Stages				Response
	I	II	III	IV	
BG1	**29.6±1.5a**	6.0±0.6b	6.2±0.2c	2.6±1.5d	54.8±2.6
1/2 MS	17.6±1.5 c	24±0.6a	21.6±0.5a	9.2±0.2d	63.3 ±2.6b
MS	9.6±1.5d	12±0.6b	**33.6±0.5a**	**10.2±0.2c**	**70.8±3.6a**
BM	13.6±1.5c	18±0.6b	22.6±0.5a	6.1±0.3d	65.9±2.5b

Means followed by the same letter are not significantly different according to Tukey test (P≥0.005). Values are mean of ± SE of three experiments with ten replicates/ experiment. ANOVA test high significant at 5% level. Stages of development of protocorms (I) Protocorm with pointed apex (II) Leaf initiation (III) and Root initiation (IV).

Maximum seed germination post cryopreservation was recorded in BG1(Figure 1) medium when compared to other media *viz,* MS, ½ MS and BM media tested (Figures not shown for other media). Composition of the medium plays a crucial role in influencing in-vitro seed germination. Morphologically, protocorm colour in modified BG1 medium was light green to whitish as seen in Figure 3c. There are several reports on certain orchid seeds requiring higher salt content medium for germination (Dohling *et al.,* 2008; Paul *et al.,* 2011). On analysing BG1 medium it was found that it had low salts contents in its composition thereby making it more suitable for the seeds of *P. Villosum* seeds to germinate. This is also supported by reports (Pierik *et al.,* 1988; Nikabadi *et al.,* 2014; Zeng *et al.,* 2015) that suggest in few selected orchids the seeds require even less than half of both micro and macronutrients for initiating germination in seeds. Another key factor in the composition of BG1 which varied was in its source of carbohydrates as glucose. Glucose being the simplest form of carbohydrates may have adhered to the easy assimilation by the seeds to germination (Nikabadi *et al.,* 2014). The beneficial effects of glucose for early germination has been reported by Traore and Guiltinan (2006) and Long *et al.* (2010). Germinated seeds were subcultured in different media *viz,* BG1, ½ MS, MS and BM media for conversion into protocorms and seedlings followed by subculture in optimum media incorporated with different growth regulators for further growth and development of the seedlings. Development of different stages (I and II) of protocorm and stages (III and IV) for seedling development was assessed. It was observed that maximum number of protocorms was retained in BG1 medium perhaps the best seed germination being on the same medium, however conversion into seedlings was favoured in MS (Table 1).



	Control	5N+10B	10N+5B	15N+15B	20N+10B	25N+5B	5I+5B	5I+20B	15I+20B	20I+10B	25I+10B
Shoot No.	2	3.1	2.9	2.8	2.3	2.5	3.7	5.1	3.1	2.9	2.8
Root No.	2.1	2.3	2.1	2.3	2.3	2.4	3.1	4.2	3.9	3.1	3.1
Shoot length (cm)	3.5	2.1	2.6	4.1	4	2.9	3.8	6.7	2.6	4	2.9
Root length (cm)	2.1	1.4	0.8	1.1	2.1	1.5	3.4	4.1	2.8	2	2.2

Figure 2: Seedling growth of *P. villosum* in MS medium incorporated NAA (N) + BAP (B) and IAA (I) + BAP in different concentration µM of plant growth regulators. Data recorded after 60 DAI. Mean followed by the same letter are not significantly different according to Tukeys test (P≥0.005)

Protocorm development into seedling is a slow sequential process as was also reported by Robinson *et al.* (2009). The overall conversion percentage till root stage was significantly different in MS medium from that of other media tried (Table 1). Therefore, MS medium was regarded optimal medium for seedling growth. This also shows that the carbohydrate requirement of the protocorm shifts from glucose to sucrose. This implies that the nutrient requirement of the protocorm varies during the different stages of development of shooting and rooting as also suggested in *Cypripedium* (Bae *et al.,* 2010). Further, results of overall seedlings of *P. villosum* showed that, the best growth and development was recorded in MS medium supplemented with 20 µM BAP+5 µM IAA, with maximum average number of shoots (4.5 cm), shoot length (6.5 cm), average root number (4.0 cm) and root length (4.1 cm) recorded after 60 DAI (Figure 2). This shows that the nutrient requirement for different stages of development varies (Nadarajan *et al.,* 2011; Zeng *et al.,* 2013). In the present study, incorporation of BAP and IAA was found stimulatory for seedling growth of *P. villosum* which is in similar to earlier reports (Long *et al.,* 2010; Zeng *et al.,* 2012; Chen *et al.,* 2015) in which BAP and NAA in combination was effectively used for in-vitro propagation of several orchids. It was observed that with further increase in concentration of both the cytokinins and auxins, seedling growth of *P. villosum* was inhibited. Nagaraju *et al.* (2003) had also reported that higher concentrations of cytokinins and auxins do show inhibitory effect on growth of orchids. The concentration of exogenous cytokinins and auxins and its synergistic effects in combinations under balanced condition, varies between species to species (Hossain, 2008; Hossain *et al.,* 2010; Long *et al.,* 2010; Roy *et al.,* 2011; Zeng *et al.,* 2012). The optimized medium with growth regulator was further used for subculturing at interval of 35 days.

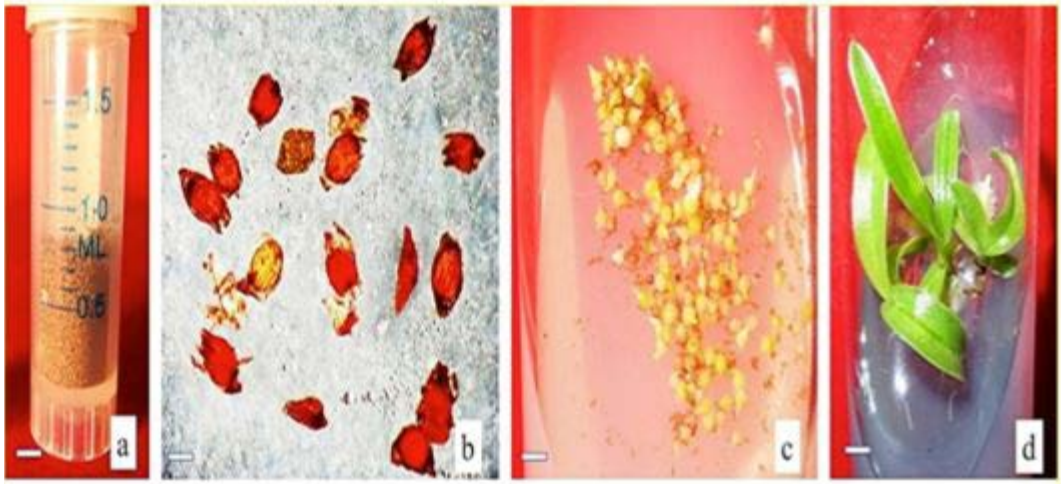


Figure 3: Seed storage (a) Seeds >240DAP of *P. villosum* stored in cryovials; (b) 1% TTC tested staining mature seeds after storage in -196°C liquid nitrogen for 180 days; (c) germination in BG1 medium; (d) seedling growth in MS medium supplemented with 5 µM BAP +10µM IAA.

Thus, in the present study on *P. villosum*, we report on seed storage method recommending in LN (-196 °C) to be most effective in not only storing and retaining the seed viability but also this process helps in protecting the seeds against pathogen attack as has also been reported in other orchid species (Popova *et al.,* 2016; Diengdoh *et al.,* 2017; Schofield *et al.,* 2018).

We have also reported the complete protocol till seedling development of post LN storage of *P.villosum* seeds. Hence the same protocol can be applied for many such threatened species of *Paphiopedilum* as well as other orchid species with some modification.

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