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In Vitro Propagation of *Paphiopedilum spicerianum* (Reichb. F.) Pfitz.

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ABSTRACT

The goal of this study was to investigate various factors affecting germination *in vitro* and to establish a protocol for propagation of *Paphiopedilum spicerianum* which would provide information on various events of seed germination and seedling development thus helping to propagate and conserve this species. Capsules were harvested at two different stages of development. Six asymbiotic orchid seed germination media (Knudson C (KC), modified Knudson C (MKC), Terrestrial orchid medium (BM), modified Terrestrial orchid medium (BM1), Vacin and Went (VW) and Nitsch and Nitcsh (N)) were examined for their effectiveness in promoting seed germination, protocorm and seedling development of *P. spicerianum*. Besides checking the efficacy of different media, the effect of capsule maturity level, photoperiod (0/24 h; 12/12 h L/D), activated charcoal, plant growth regulators [6-benzyladenine (BA) and α-naphthalene acetic acid (NAA)] on seed germination was also assessed. The seeds from undehisced green capsules germinated with better frequency than seeds from mature burst capsules. Germination occurred regardless of media type. Amongst all media tested, highest germination percentage (62.75 ± 2.63%) was achieved in BM1 and activated charcoal (AC) under continuous darkness. The addition of NAA (1.5 mg l^{-1}) to medium resulted in the early formation of seedlings within 21.05 ± 0.05 weeks. Higher concentration of BA reduced the percentage of seed germination. After germination for 8 weeks in total darkness at the protocorm stage, a shift from darkness to light conditions (12/12 h L/D) was required for differentiation of protocorms into seedlings. Chlorophyll development was a post-protocorm phenomenon in the cultures. The current study has the potential to assist with the future development of *ex situ* conservation of this endangered species by producing innumerable viable seedlings.

Keywords: asymbiotic, endangered, *in vitro* culture, seedlings, terrestrial orchid
Abbreviations: AC, activated charcoal; **BA**, 6-benzylaminopurine; **BM**, Terrestrial orchid medium; **BM1** modified Terrestrial orchid medium; **KC**, Knudson C medium; **MKC**, modified Knudson C medium; **NAA**, α-naphthalene acetic acid; **N**, Nitsch and Nitsch medium; **VW**, Vacin and Went medium

INTRODUCTION

The genus *Paphiopedilum* Pfitzer comprises about 80-85 species in the world. It includes a number of horticulturally important species and produces a wide range of attractive varieties and hybrids (Cribb 1998). Geographically, the genus is distributed in tropical Asia to the Pacific islands, with some species extending to subtropical areas (Liu *et al*. 2011). Paphiopedilums are among the most widely cultivated and hybridized of orchid genera. Their wild populations are under threat of extinction. The major causes of depletion of their genetic diversity are habitat conversion to residential areas or agricultural lands, habitat mismanagement and fragmentation, illegal commercial collections, and increased use of fertilizers. Currently, they figure among endangered orchids in appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES 2012). Integrative multidisciplinary approaches are required for conservation (*in situ* and *ex situ*) and sustainable use of critically endangered and floriculturally important *Paphiopedilum spicerianum*. Efforts are needed to conserve and eco-restore the species by developing effective conservation techniques. Germination is a series of complex physiological processes during which takes place reactivation of metabolic energy, protein synthesis, transport of nutrients, etc. Orchid seeds are microscopic, non-endospermic and enclosed within a transparent testa cover. They are produced in large numbers but in natural habitat very few of them germinate successfully, countless perish away since they require suitable mycorrhizal associations and environmental conditions to accomplish germination. Under these circumstances use of *in vitro* protocols has proven as a successful approach for *ex-situ* conservation and reintroduction of endangered orchids (Decruse *et al.* 2003; Sarasan *et al.* 2006) and many other species of commercial importance. With a view to conserve these marvels of nature which are extensively exploited for the floriculture trade, *P. spicerianum* was selected for this study.

Endemic to the north-eastern Indian Himalayas (Hajra and De 2009), *P. spicerianum* grows in temperate climates within an altitude of 300-1,300 meters. Due to its beautiful blossoms, the species ranks high among the horticulturally most important genotypes. Its population's decline is attributed to many factors (as stated above), largely to over-exploitation for its whole plant by commercial growers for the floriculture trade. Vegetative propagation is quite slow and time consuming as only one new growth per plant is produced in a year. Their seeds are also considered hard to germinate *in vitro* (Pierik *et al*. 1988) since they have stringent requirements for germination, moreover their explants are also difficult to maintain in culture (Arditti and Ernst 1993) and survival rates of deflasked seedlings even more so (Rasmussen 1995). There are a few reports of asymbiotic seed germination of *Paphiopedilum* species (Lucke 1971; Ernst 1974, 1980; Flamee 1978; Stimart and Ascher 1981; Pierik *et al*.1988; Tay *et al*. 1988; Long *et al*. 2010; Ng *et al*. 2010; Zeng *et al*. 2012).

Immediate conservation measures are required to mass propagate and save this species. Thus, an attempt has been

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made in this study to conserve its gene pool from getting extinct through tissue culture technique using seeds from undehisced green capsules and mature burst capsules.

Terrestrial orchid species prefer to germinate on a low salt medium and the nitrogen source plays a critical role in seed germination (Fast 1982; Van Waes and Debergh 1986; Rasmussen 1995; Kauth *et al*. 2006; Stewart and Kane 2006). Most of the orchid asymbiotic seed germination media are composed of similar components. The only difference lies in available nitrogen source. Nitrogen source is known to influence enzyme synthesis during initial stages of seed development (within the developing protocorms), thereby ensuring that organic nitrogen can be more readily utilized by young protocorms as available amino acids which may bypass certain steps of the nitrogen assimilation process, i.e. prompt production of nitrate reductase following seed imbibition (Ragahavan and Torrey 1964; Malmgren 1992). The nutrient regime for orchid culture is species specific and no single culture medium is universally applicable for all orchid species.

Here, we concentrate on the ability of immature and mature seeds to germinate, which is one of the most important aspects of orchid biology, by conducting a series of experiments for establishing a micropropagation protocol. In particular, we verified the following: 1) influence of capsule stage, 2) suitable seed germination medium, 3) culture conditions, 4) efficacy of plant growth regulators, and 5) effect of activated charcoal on germination of seeds and their successive development into seedlings.

MATERIALS AND METHODS

Plant collection and greenhouse maintenance

Paphiopedilum spicerianum plants and their capsules were collected from a commercial grower of Darjeeling district, West Bengal, India (latitude range: 26° 31′ - 27° 13′ N; longitude range: 87° 59′ - 88° 53′ E). Plants were replanted in pots (27.5 cm × 22.4 cm in diameter) containing soil as a substrate, and maintained in a greenhouse under natural light conditions and 22°C/20°C (day/night temperature). A voucher specimen (Herbarium number NIP - 154) was deposited in the herbarium of the Department of Natural Products, National Institute of Pharmaceutical Education and Research, Mohali, Punjab, India. The capsules were allowed to grow and mature. They were plucked and sacrificed at different time intervals, to assess the effect of undehisced (green capsules) and dehisced (brown capsule with dry cover) capsules on asymbiotic seed germination. Capsules were collected, brought to the laboratory and prepared for inoculations onto media on the same day.

Surface sterilization

Green capsules were first scrubbed with a soft brush in running tap water and later with dish washing liquid to remove any debris from the surface of capsules then rinsed thoroughly in water. They were swabbed with ethyl alcohol under a sterile (laminar) hood and surface sterilized with 0.1% mercuric chloride ($HgCl_2$; Qualigens, Mumbai, India) in an aqueous solution containing 1-2 drops of liquid soap as a wetting agent for 7 min. Capsules were rinsed 2-3 times with sterilized distilled water to remove any traces of $HgCl_2$ left on their surface. Thereafter, the capsules were flamed on a burner and seeds were scooped out into a Petri dish by making a longitudinal slit and inoculated onto different media.

The seeds from dehisced capsule were collected on thin glazed paper. Inside laminar air flow cabinet, carefully they were poured in a flask and given a treatment of sodium hypochlorite 4% (Merck, Mumbai, India) for 10 min. Finally, whole mixture was filtered. The sterilized seeds were washed with sterile distilled water two times and inoculated on different media.

Culture media for *in vitro* seed germination and autoclaving

The seeds were inoculated on different media combinations such as Knudson C (1946) medium and its modified version (MKC), Terrestrial orchid medium (BM; Van Waes 1984) and modified Terrestrial orchid medium (BM1; Van Waes 1984), Vacin and Went (1949) medium and Nitsch and Nitsch (1969) medium with and without AC (0.2%). The effect of BA (1.0, 1.5, 2.0 mgl^{-1}; Himedia), and NAA (1.0, 1.5, 2.0 mgl^{-1}; Hi-media) was also assessed on seed germination and seedling development. The pH of medium was adjusted according to the medium requirement with 1 N NaOH or HCl before adding agar and autoclaving. Media were dispensed in test tubes of size (25 mm × 150 mm) and autoclaved at 121°C at a pressure of 1.06 kg cm^{-2} for 15 min. Autoclaved medium was kept at 37°C to check any further contamination.

Inoculations and incubation conditions

The inoculations were done under aseptic conditions in a laminar air flow cabinet. The culture vessels were incubated at 25 ± 2°C under two different light regimes i.e. continual darkness of 0/24 h L/D and 12/12 h L/D photoperiod illumination of 3,500 lux light intensity (Fluorescent tubes, 40W; Philips India Ltd, Mumbai, India). Four replicates were used for each experiment and to check the reproducibility the experiments were repeated twice. The cultures were sub-cultured as and when required.

Observations and statistical analysis

The cultures were examined regularly using binocular microscope (Olympus SZX10, Tokyo, Japan) and data recorded accordingly. The results were expressed as means ± SD of four replicates. The results were tested using one-way ANOVA test and were analyzed using Tukey's Multiple Comparison ($P \leq 0.05$) using SPSS version 16.0 (SPSS Inc. Chicago, US).

Percent germination

Nearly after six weeks of inoculation, some of the seeds were scoped out and dispersed in a drop of water on a glass slide and observed under light microscope. Germination was calculated employing the following formula: Germination (%) = (Number of enlarged seeds showing swelling of the embryo × 100) / Total number of seeds. Germination was considered to have occurred when the embryo emerged from the testa cover. Once the spherules were formed, observations were recorded at an interval of one week to trace different stages of development of cultures. These were observed using a stereozoom microscope (Nikon, H600L, Tokyo, Japan).

RESULTS

Seed germination

In general, the seeds consisted of undifferentiated cells enclosed within a hyaline seed coat. The process of germination started after six weeks of inoculation with swelling of the embryo (**Table 1**). The swollen seed emerged out of seed coat as spherule. The spherule grew in size while still attached to the seed coat (**Fig. 1A**). In another 4 weeks, these spherules started differentiating into conical, non-chlorophyllous protocorms (**Fig. 1B**) (no chlorophyll developed in darkness). At the initial stages of protocorm formation a shoot tip (1st leaf primordia) was evident at one end Later more rhizoids developed all around at the basal portion of the non-chlorophyllous protocorm and second leaf primordia was also formed (**Fig. 1B**). The first signs of chlorophyll development were apparent after 2nd leaf primordia initiation in the cultures, when the cultures were shifted from darkness 0/24 h L/D to 12/12 h L/D photoperiod (**Fig. 1C**). The roots were initiated soon after at the base of the leaf and seedlings were formed. On prolonged culturing under same photoperiodic condition, the protocorms multiplied in the cultures (**Fig. 1D**), later these protocorms were transferred to fresh medium where they transformed into complete plantlets.

Table 1 *In vitro* seed germination and seedling development of *Paphiopedilum spicerianum* in BM and BM-1 medium.

Medium	Germination %	Initiation of germination (weeks)	Development of protocorm (weeks)	Development of leaf (weeks)	Development of root (weeks)	Seedling formation (weeks)
BM	30.00 ± 1.07 ^{bdefgh}	7.95 ± 0.31 ^{bcdefgh}	13.10 ± 0.11 ^{bcefgh}	16.25 ± 0.50 ^{bcdegh}	20.50 ± 1.00 ^{bdegh}	26.62 ± 0.25 ^{bdegh}
+$BA_{0.2}$*	62.75 ± 2.63 ^{acdefgh}	6.12 ± 0.09 ^{acdefgh}	10.00 ± 0.00 ^{acdefgh}	13.00 ± 0.00 ^{acdefgh}	17.00 ± 0.00 ^{abdefgh}	23.80 ± 0.35 ^{acdegh}
+BA_1	29.25 ± 0.50 ^{bdefgh}	10.50 ± 0.57 ^{abh}	13.92 ± 0.29 ^{abdegh}	17.05 ± 0.10 ^{abdeg}	21.00 ± 0.00 ^{bdefgh}	27.85 ± 0.50 ^{abdefgh}
+$BA_{1.5}$	19.50 ± 0.57 ^{abcf}	10.75 ± 0.50 ^{abh}	13.05 ± 0.10 ^{bcefgh}	18.05 ± 0.10 ^{abcfgh}	24.18 ± 0.12 ^{abcef}	29.08 ± 0.09 ^{abcfg}
+$BA_{2.0}$	19.51 ± 0.23 ^{abcf}	10.75 ± 0.00 ^{abh}	16.15 ± 0.19 ^{abcdfgh}	18.05 ± 0.05 ^{abcfgh}	24.18 ± 0.09 ^{abcfg}	29.10 ± 0.08 ^{abcfg}
*+NAA_1	36.75 ± 2.63 ^{abcdegh}	10.00 ± 0.00 ^{abh}	14.00 ± 0.00 ^{abdegh}	16.75 ± 0.50 ^{bdeg}	20.00 ± 0.00 ^{bcdefg}	24.50 ± 0.57 ^{acdegh}
*+$NAA_{1.5}$	20.00 ± 0.00 ^{abcf}	10.25 ± 0.50 ^{abh}	12.00 ± 0.00 ^{abcdefh}	15.00 ± 0.00 ^{abcdefh}	18.00 ± 0.00 ^{abcdefh}	21.05 ± 0.05 ^{abcdegh}
*+NAA_2	19.75 ± 0.50 ^{abcf}	12.75 ± 0.50 ^{abcdefg}	17.10 ± 0.11 ^{abcdefg}	20.50 ± 0.00 ^{abcdefg}	24.25 ± 0.50 ^{abcfg}	29.15 ± 0.19 ^{abcfg}

BM – Terrestrial orchid medium; * = modified terrestrial orchid medium (BM-1); Values in a column with the same superscript are not significantly different at $P \leq 0.05$ according to Tukey's test; Subscript is concentration of growth regulators = mg l^{-1}.

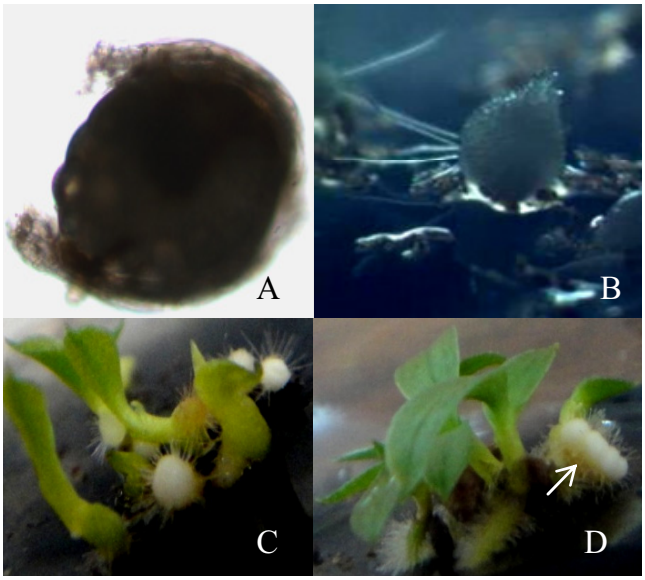

Fig. 1 ***In vitro*** **seed germination and seedling development in** ***Paphiopedilum spicerianum*****.** (**A**) Spherule attached to seed coat; (**B**) Non-chloro-phyllous protocorm with rhizoids at the basal portion; (**C**) Cultures at leaf stage growing under 12/12 h L/D photoperiod; (**D**) Seedlings with proto-corm multiplication.

Table 2 The effect of capsule stage on germination percentage of *Paphiopedilum spicerianum in vitro.*

Capsule stage	Germination %
Dehisced	17.75 ± 0.03 ^{a}
Undehisced	62.75 ± 2.63 ^{b}

Values in a column with the same superscript are not significantly different at $P \leq 0.05$ according to Tukey's test

Effect of capsule stage on seed germination

In this investigation time of capsule harvest emerged as one of the important parameters to accomplish seed germination. The germination percentage varied significantly with respect to the stage of the capsule. Higher number of seeds (62.75 ± 2.63%) germinated from the capsules harvested at green undehisced (immature) condition (**Table 2**). The seeds collected from the capsules, which was harvested at a stage when capsule sutures started splitting, showed much delayed germination with declined germination percentage; only 17.75 ± 0.03% of seeds germinated.

Effect of basal media, incubation photoperiod and activated charcoal on seed germination and seedling development

The seeds (from un-dehisced green capsules) germinated in all basal media irrespective of the photoperiods to which they were subjected. The prompt and highest percentage of seed germination (62.75 ± 2.63%) was observed in modified terrestrial orchid medium (BM + BA; 0.2 mg/l) under complete dark (0/24 h L/D) incubation conditions (**Table 3**). In terrestrial orchid medium, nearly 30.00 ± 1.07% seeds germinated. For successful conversion of protocorms into seedling development, a shift was necessary from 0/24 h L/D photoperiod to 12/12 h L/D illumination. Otherwise if kept under continual dark the protocorms turned brown and perished soon after. In modified KC medium, only 25.00 ± 0.81% seeds germinated under 0/24 h (L/D) photoperiod. In media such as KC, VW and N medium the onset of germination was much delayed and percentage of germinating seeds was reduced under both light (12/12 h L/D) and dark (0/24 h L/D) photoperiods particularly in N and VW medium, the germination was exceptionally low. In N medium under 12/12 h L/D photoperiods in with/without AC enriched medium only 6.00 ± 1.70/4.75 ± 0.50% seeds germinated respectively (**Table 3**) whereas under 0/24 h L/D photoperiod condition, the germination percentage was little higher. Similar situation was observed with VW medium. The protocorms in these media started turning brown. These were shifted on to BM-1 medium where they developed into seedlings and those which were left in the same culture media did not develop further and died. Additional activated charcoal in the media favoured early germination of the seeds and their development in to seedlings, particularly in modified terrestrial orchid medium the frequency of germinating seeds was much enhanced as compared to medium devoid of activated charcoal.

Effect of BA and NAA on seed germination and seedling development

Presently, the effect of BA and NAA on seed germination and seedling development was tested in BM and BM-1. A marked variation was observed in germination percentages in medium supplemented with BA at varying concentrations. BA at 0.2 mgl^{-1} (modified BM) favoured early onset of germination with highest seed germination percentage; seeds germinated with a maximum of 62.75 ± 2.63%. Additional NAA (1.5 mgl^{-1}) in BM-1 initiated early rooting and seedling formation. Seedlings complete with leaf and root was formed within 21.05 ± 0.05 weeks (**Table 1**). Higher concentrations of BA i.e. 1.5 and 2.0 mgl^{-1} and NAA 2 mgl^{-1} significantly reduced germination percentages. While examining the cultures an interesting observation was made in NAA treated cultures. At the protocorm surfaces lump like structures were seen. After 4 weeks of culture these clubbed structures developed septate and unbranched rhizoids at the lower surfaces.

DISCUSSION

In vitro asymbiotic seed germination has a great potential as a propagation method to produce viable seedlings of rare, critically endangered and commercially important orchids and other species. Success of asymbiotic germination is dependent upon identifying correct stage of capsule at harvesting time, suitable culture medium, growth regulator/s and culture conditions *in vitro*. In this study, seed germination of *P. spicerianum* was influenced by maturity level of capsule, photoperiod, germination media, growth regulators

Table 3 The effect of media, activated charcoal and photoperiods (0/24 h; 12/12 h L/D) on in vitro seed germination of *Paphiopedilum spicerianum*.

Medium	Germination % under 0/24 h (L/D) photoperiod		Germination % under 12/12 h (L/D) photoperiod	
	-AC	+AC	-AC	+AC
BM	27.50 ± 2.50^{bdef}	30.00 ± 1.07^{bdef}	22.50 ± 1.44^{cef}	21.25 ± 1.25^{ef}
BM-1	42.25 ± 1.31^{acef}	62.75 ± 2.63^{acdef}	25.00 ± 0.00^{cef}	26.25 ± 1.25^{cef}
KC	22.25 ± 2.62^{bdef}	25.00 ± 0.40^{bdef}	15.00 ± 2.04^{abef}	17.50 ± 0.10^{bef}
MKC	25.50 ± 0.81^{bef}	46.25 ± 1.25^{abcef}	18.75 ± 1.25^{ef}	21.25 ± 0.75^{ef}
N	6.25 ± 1.25^{abcd}	11.00 ± 0.57^{abcd}	4.75 ± 0.50^{abcd}	6.00 ± 1.70^{abcd}
VW	10.50 ± 0.50^{abcd}	13.25 ± 0.47^{abcd}	7.50 ± 1.43^{abcd}	8.00 ± 0.50^{abcd}

L – Light, D – Dark; Values in a column with the same superscript are not significantly different at $P \leq 0.05$ level according to Tukey's test.

and AC.

The seeds of *P. spicerianum* procured from undehisced (immature) green capsule germinated readily with better frequency than those collected from dehisced brown capsule, due to their metabolically awakened embryos. While the earliest stage at which the immature seeds acquire ability to germinate could not be tested in this species. A review of literature reveals that maturity related progressive decline of percentage of seed germination is attributed to induction of dormancy due to accumulation of inhibitory substances and rapid loss of viability (Stoutamire 1974; Linden 1980; van Waes and Debergh 1986; Miyoshi and Mii 1988; De Pauw and Remphrey 1993; Rasmussen1995).

An investigation made by Vasudevan and van Staden (2010) in *Dendrobium nobile* and Lee *et al*. (2008) in *Phalaenopsis amabilis* var. *formosa*, confirmed a correlation between internal organization of immature seeds and the germinating abilities of seeds which indicated that discontinuous cuticle layer enveloping the embryo proper, presence of gaps created by the cellular degeneration of the inner integument, and the absence of secondary wall thickenings in the outer integument, all these morphological changes of seed integuments plays an important role in maximizing the germination percentages of the immature seeds. As the embryos approaches towards maturity, embryo dehydrates due to the development of hydrophobic carapace sheath and lignification of the cell walls of testa (Rasmussen 1995). Harrison (1977) correlated the inability of the mature seeds to germinate with ease with, lack of an appropriate metabolic machinery (glyoxysome bodies) which is capable of utilizing their own lipidaceous food reserves. According to Lee *et al*. (2007) a negative correlation between the concentrations of endogenous abscisic acid (ABA) and rates of seed germination infers that high endogenous ABA concentrations are always associated with poor seed germination. All these features strongly explain the possible reasons of reduced germination percentages of mature seeds of *P. spicerianum* also.

Although, in our experiment, seed germination occurred regardless of photoperiod in all the media treatment similar to those reported earlier in *Paphiopedilum sukhakulii* (Tay *et al*. 1988), *Calopogon tuberosus* (Kauth *et al*. 2006), *Bletia purpurea* (Dutra *et al*. 2008), *Phragmipedium humboldtii*, *P. longifolium* and *P. pearcei* (Muñoz and Jiménez 2008) dark treatment (0/24 h L/D) favoured early onset of germination with better germination percentage in contrast to light (12/12 h L/D) conditions. Similar result was obtained by Zeng *et al*. (2012) in *Paphiopedilum wardii* where darkness promoted seed germination percentage in comparison to light photoperiod. Literature studies reveal that, in many terrestrial orchids, seeds require dark conditions to initiate germination process as observed in *Paphiopedilum ciliolare* (Pierik *et al*. 1988), *Paphiopedilum sukhakulii* (Tay *et al.* 1988), *Cephalanthera falcata* (Yamazaki and Miyoshi 2006). The inhibitory effect of illumination of terrestrial species has been stated to be a protective mechanism (Stoutamire 1974). In nature *P. spicerianum* inhabits shadier localities and the seeds germinate under low light intensity. The species simulated similar kind of environment *in vitro* to accomplish maximum germination. This appears to be the one of the possible reason for their maximum germination under dark conditions as compared to light regimes in the cultures. Photoperiod response in the germination of terrestrial orchid species varies and are intrinsic traits (genotype) for individual species (Arditti *et al*. 1981). Besides stimulatory and inhibitory effects of illumination and continual darkness on terrestrial orchid seed germination there are findings which show that light and dark photoperiods do not have any significant effect over germination as observed in *Goodyera oblongifolia* (Arditti *et al*. 1981), *Alplectrum*, *Spiranthes*, *Cypripedium* (Oliva and Arditti 1984), and *Phragmipedium humboldtii*, *P. longifolium* and *P. pearcei* (Muñoz and Jiménez 2008). Results of this study and descriptions in previous reports indicate that inhibitory as well as promotive effects of light/dark on the seed germination and the seedling development of the terrestrial orchid species are intrinsic traits (genotype) for each species.

The chlorophyll development was observed as a post-protocorm phenomenon in our cultures. The protocorm that developed in dark were achlorophyllous; when shifted under light conditions, the chlorophyll synthesis began and they turned green as observed in other orchid species (Arditti and Ernst 1993), *Phragmipedium humboldtii*, *P. longifolium* and *P. pearcei* (Muñoz and Jiménez 2008). According to Stoutamire (1974) the non-chlorophyllous protocorms characterize the species growing in well-drained soils. The present species inhabits ground soils, and non-chlorophyllous protocorms in these may be a genetic attribute.

Several macro- and micronutrients affect the growth and development of orchids, but major effect is seen with nitrogen (Rasmussen 1995). Nitrogen is an essential component of mineral nutrient media used for plant growth and development, which occur in the oxidized form as nitrate or in the reduced form as ammonium, bound in inorganic salts or in organic compounds. Many species show a preference for nitrogen in reduced form as ammonium (Ichihashi and Yamashita 1977). In nature orchid soils are naturally poor in readily absorbable nitrogen, but the humus content of the soil gives a high concentration of organic nitrogen accessible to fungi which provides an organic form of nitrogen to germinating seeds of orchids (Rasmussen 1995). High concentrations of inorganic nitrogen affect the germination negatively (van Waes 1984; Rasmussen 1995). Many species show a preference for nitrogen in reduced form as ammonium (Ichihashi and Yamashita 1977). Malmgren (1992) found that germination rates of temperate terrestrial orchid species were higher in media containing amino acids. Stewart and Kane (2006) stated that an organic form of nitrogen could influence *in vitro* growth and development of *Habenaria macroceratitis*. In our experiment, nitrogen source differed in all the media screened. KC, N, VW, and BM contained inorganic form of nitrogen whereas modified terrestrial orchid medium (BM1) contained organic form of nitrogen as hydrolysed casein, L-glutamine. In an earlier study, addition of peptone significantly increased seed germination of *Paphiopedilum* and *Vanda* species and lowered that of *Platanthera clavellata* (Curtis 1947). Modified terrestrial orchid medium proved optimum in eliciting maximum germination of seeds and early onset of germination due probably to the activation of nitrate reductase activity at very early stage because of the presence of organic nitrogen compounds as suggested by Dutra *et al*. (2008) in *Bletia purpurea*.

Terrestrial orchid species are dependent on mycorrhizal partners during germination and seedling development in

nature, as the endophyte provides necessary elements for germination such as cytokinins and enzyme precursors (Arditti 1979; Hadley and Peggi 1989). Cytokinins are most important growth regulators affecting *in vitro* germination (Harvais 1982). They are extensively used in orchid seed germination media (Arditti and Ernst 1993). Their influence on seed germination is species specific. In our study, BA 0.2 mgl^{-1} in terrestrial orchid medium induced early onset of germination with maximum germination percentage but its higher concentrations lowered germination percentages and delayed onset of germination. Less germination occurred in BM without BA, which could have occurred due to inadequate levels of BA in the seeds that did not germinate similar to the situation observed in *Cypripedium candidum* (De Pauw *et al*. 1995). Since in orchid seeds the reserves are present in the form of lipid droplets (Arditti and Ernst 1984), it is suggested that possible role of cytokinin in the germination medium is utilization of complex carbohydrates, without which germination could not accomplish (*cf*. De Pauw *et al*. 1995).

In most terrestrial species it is generally observed that auxins do not have any pronounced effect on seed germination (Van Waes 1984; Rasmussen 1995). In the present experiment, NAA (1.5 mgl^{-1}) in modified BM, however, delayed onset of germination but favoured early seedling development. Our results are similar to those reported earlier by Harvais (1982) in *Cypripedium reginae* where better growth of seedlings was observed in a combination of auxin and cytokinin. Generally, auxins stimulate root formation and cytokinins enhance shoot development and cell division. Similarly, in our experiment also, *P spicerianum* seedlings had accelerated development in the presence of auxin and cytokinin combinations.

Activated charcoal is known for its proven efficacy due to the property of adsorption of inhibitorysubstances (phenolics) exuded by the cultures. Presently, germination response was significantly higher in activated charcoal enriched medium. Earlier researchers also reported its efficacy in *Paphiopedilum* and *Phalaenopsis* seedling cultures, (Butcher and Marlow 1989; Hicks and Lynn 2010) and in other terrestrial and lithophytic orchid species (Nadarajan *et al*. 2011). A study made by Pacek-Bieniek *et al.* (2010) while examining the influence of AC on seed germination and seedling development indicated its positive effects in enlarging protocorm size, in intensive development of leaf and maximum aerial root growth in *Zygostales grandiflora*. AC plays an important role in orchid seed germination (Moraes *et al*. 2005).

In this experiment, there was a differential requirement with respect to culture conditions as well as culture medium at the time of initiation of seed germination and seedling development. This differential nutritional requirement for germination and seedling growth has also been observed in *Paphiopedilum sukhakulii* (Tay *et al*. 1988), *Calopogon tuberosus* (Kauth *et al*. 2006, 2008), *Bletia purpurea* (Dutra *et al*. 2008), *Phragmipedium humboldtii*, *P. longifolium* and *P. pearcei* (Muñoz and Jiménez 2008), terrestrial and lithophytic orchid species (Nadarajan *et al*. 2011).

CONCLUSION

Therefore, we strongly emphasize up on the need to consider the physical and chemical factors which determine maximum seed germination for conservation of this endangered orchid species and point out that capsule stage is very important to achieve better seed germination and the capsules should be harvested when they still hydrated (undehisced/immature) and green in colour. From this study it is concluded that best medium for seed germination of *P spicerianum* is BM1 containing activated charcoal under 0/24 h L/D photoperiod. For differentiation of protocorms into seedlings, the cultures should be given 12/12 h L/D photoperiod treatment. Optimum medium for seedling growth is BM-1 supplemented with activated charcoal and NAA (1.5 mgl^{-1}) under 12/12 h L/D illumination.

In vitro culture techniques assist in germplasm conservation of endangered orchid species of floricultural importance. There is immense scope for further development of *in vitro* and *ex vitro* methodologies to save germplasm of this valuable species. Further research should focus on improving efficiency of *P. spicerianum* seed germination, especially investigating the use of mycorrhizal fungi, pollinator relationships, on the acclimatization, and on the establishment of *in situ* protocols for conservation of this floriculturally significant rare, endangered species, other threatened orchids and species of therapeutic and commercial importance.

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