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ORIGINAL RESEARCH PAPER

# Asymbiotic seed germination and multiplication of an endangered orchid – *Paphiopedilum venustum* (Wall. ex Sims.)

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## Abstract

The present study was intended to facilitate ex situ conservation of *Paphiopedilum venustum*, a highly floriferous endangered terrestrial orchid species. A protocol was established for in vitro propagation and shoot multiplication. The cultures were initiated through asymbiotic seed germination technique, using undehisced and dehisced capsules. Four defined asymbiotic orchid seed germination media (terrestrial orchid medium, modified terrestrial orchid medium, Malmgren modified terrestrial orchid medium, Knudson C medium) were evaluated for their effectiveness in achieving maximum seed germination and early seedling development. The effect of darkness and 12-h photoperiod was also tested. Optimum seed germination, i.e., 82.7% was achieved on modified terrestrial orchid medium under a 12-h photoperiod using seeds from undehisced capsules. Shoot multiplication was accomplished using organic [peptone (1.0, 2.0 g $L^{-1}$)] and inorganic [banana homogenate (10, 20, 30 g $L^{-1}$) and potato powder (5.0, 10 g $L^{-1}$)] growth supplements. Peptone at 1.0 g $L^{-1}$ was the most effective in multiplying the shoots. Plantlets were acclimatized in the greenhouse with 80% survival frequency.

## Keywords

organic supplements; peptone; slipper orchid; terrestrial orchid

## Introduction

The genus *Paphiopedilum* (Orchidaceae), known as "slipper orchids", is popular as cut-flowers due to their high aesthetic value and longevity. *Paphiopedilum* encompasses 80 species worldwide [1] distributed in tropical Asia including the Pacific islands, with some species extending to subtropical areas [1]. It includes a number of commercially important species that produce a wide range of attractive hybrids [2]. The major reason for the depletion of genetic diversity of *Paphiopedilums* is habitat destruction through the conversion of forest areas into residential areas, agricultural lands and illegal commercial collections for the trade. As a consequence, the genus is at the verge of extinction and included among other endangered orchids, which are listed in the appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora [3]. Therefore, an attempt was made to conserve the gene pool of this genus by selecting a species, i.e., *Paphiopedilum venustum*.

*Paphiopedilum venustum* is an endemic terrestrial species of the Himalayan ranges. It grows at an altitude of 500–1500 m, in shade and moderate light conditions. In the

**Fig. 1** Plant of *Paphiopedilum venustum*.

Indian subcontinent, the species is distributed in India (Arunachal Pradesh, Meghalaya, Sikkim) Nepal, Bhutan, and Bangladesh [4]. It is extremely popular, as an indoor plant worldwide, due to its dark-green mottled foliage (Fig. 1). Owing to its beautiful blossoms, the species ranks highly among the horticulturally important genotypes. The species is a progenitor of meritorious artificial and naturally occurring hybrids of international reputation such as *P.* ×*venusto-insigne*, *P.* ×*spicero-venustum* and a hybrid between *P. farrieanum* × *P. venustum* called *P. pradhanii* [5].

The main problem associated with slipper orchids is the low seed germination percentage both in vivo and in vitro. The seeds of *Paphiopedilum* species are considered hard to germinate and in vitro cultures are difficult to achieve [6,7]. Vegetative propagation through the division of axillary buds from the mother plant is not efficient because it produces only a single new growth per year and is time consuming. Presently, efforts have been made to devise a protocol to raise seedlings by achieving optimum seed germination, multiplying the cultures and subsequently acclimatizing the plantlets under in vivo environment. In vitro culture techniques provide a practical system for clonal, rapid mass multiplication of rare, endangered, horticulturally, and therapeutically important genotypes [8–13]. Asymbiotic seed germination is an important technique for rescuing and propagating a large number of orchid species and hybrids. Few reports of asymbiotic seed germination of *Paphiopedilum* species are available [10,14–17].

Terrestrial orchids grow on a low salt medium and nitrogen source plays a critical role in initiating seed germination [18]. Nutrient regime for orchid culture is species specific and no single culture medium provides the nutritional requirements of all the orchid species. The basic culture medium requirements and the exact stage of the capsules, which support maximum seed germination varies with the species and local conditions [19].

Multiplication of shoots of *Paphiopedilum* species and its hybrids, such as *Paphiopedilum philippinense* PH 59 and PH 60 hybrids, *P. delenatii*, *P. rothschildianum*, and *Paphiopedilum* 'Delrosi' has been reported [9,20–22] . To our knowledge, no information regarding in vitro asymbiotic seed germination and micropropagation of *Paphiopedilum venustum* has been published. The objective of the present study was to establish an asymbiotic seed germination protocol using immature and mature seeds in four defined media under dark or 12-h photoperiod. Another objective was to multiply in vitro grown shoots and to acclimatize them.

## Material and methods

### Plant material

Plants of *Paphiopedilum venustum* 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). The plants were re-planted in the pots (diameter 27.5 × 22.4 cm) containing soil and leaf litter as a substrate. The pots were maintained in a greenhouse under natural light conditions and 22/20°C day/night (l/d) temperature.

### Culture medium for in vitro seed germination

Four defined asymbiotic orchid seed germination media such as BM (terrestrial orchid medium) [23], BM-1 (modified terrestrial orchid medium) [18], KC (Knudson medium) [24], MM (Malmgren modified terrestrial orchid medium) [25] were used to initiate cultures. The pH of media such as BM and BM-1 (5.4), KC (5.8), and MM (5.2) was adjusted using 1 N HCl / 1 N NaOH before autoclaving. All media were gelled with agar (Hi-media, Mumbai, India), dispensed into (25 × 150 mm) test tubes and autoclaved at 121°C at a pressure of 1.06 kg $cm^{-2}$ for 15 min. Autoclaved media were kept at 37°C for 2–3 days to check for any contamination. Seed viability was assessed using TTC (2,3,5-triphenyl-2H-tetrazolium chloride; 1%) staining method at pH 6.5 [18]. The seeds were stained for 40–48 h at 30°C in dark.

### Surface sterilization of the undehisced capsule

The capsules were first scrubbed with a soft brush in running tap water to remove any debris from their surfaces. Each capsule was rinsed thoroughly with dish-wash detergent solution. Later, the capsule was swabbed with ethyl alcohol in a flow hood and disinfected with aqueous solution of 0.05% mercuric chloride ($HgCl_2$; Qualigens, Mumbai, India) containing 1–2 drops of "Teepol" for 2–3 min. The capsule was than rinsed thoroughly with sterile distilled water. Thereafter, the capsule was incised longitudinally and the seeds were scooped out into a Petri dish.

### Surface sterilization of seeds collected from dehisced capsule

The seeds from dehisced capsules were collected on a thin glazed paper. Inside flow hood, with utmost care they were poured into a sterile flask (250-mL Erlenmeyer) and treated with sodium hypochlorite (4% NaOCl; Merck, Mumbai, India) for 15 min. The flask was capped with a tight-fitting cotton bun. The solution was filtered through a sterile funnel padded with a layer of sterile filter paper (Whatman filter paper No. 1) which was mounted in a flask (250 mL, Borosil). Disinfected seeds were washed with sterile distilled water twice.

Finally, the seeds from dehisced and undehisced capsules were spread over the media surface in as thin layer as possible, in the culture tubes with the help of a sterile spatula.

### Shoot multiplication

The shoots obtained from the asymbiotic seedlings were cut and cultured on BM-1 medium. The effect of growth supplements such as banana homogenate (bh; 10, 20, and 30 g $L^{-1}$ w/v), peptone (1.0 and 2.0 g $L^{-1}$ w/v; Hi-media, Mumbai, India) and potato powder (5.0 and 10 g $L^{-1}$ w/v; Hi-media, Mumbai, India) were tested individually for the multiplication of shoots. Bh was obtained from fruits purchased from the market. They were peeled, cut into slices and homogenized in a blender. Required quantities of the resultant pulp were weighed and added to the medium. After incorporating all the organic supplements, pH of the medium was adjusted to 5.7 prior to autoclaving.

### Inoculations and incubation conditions

The cultures vessels, inoculated with seeds, were incubated under two different light regimes darkness and 12-h photoperiod. The culture vessels for shoot multiplication were incubated under 12-h photoperiod at 40 μmol $m^{-2}$ $s^{-1}$ light intensity (fluorescent tubes, Philips India Ltd, Mumbai, India) at approximately 25°C. Eight replicates were used per treatment. To check the reproducibility of the protocol, the experiments were repeated twice. The cultures were sub-cultured into their respective fresh media when the media in the culture vessels were depleted.

### Germination of seeds

After 4 weeks of inoculation, a few seeds were taken out of the culture vessel with the help of a spatula. They were placed in a drop of water on a glass slide and observed under a microscope. Once the embryos emerged out of the seed coat and spherules were formed, the observations were taken at 1 week intervals. Different developmental stages of germinating seeds were observed and photographs were taken using a stereozoom microscope (Nikon, H600L, Japan).

 

### Acclimatization

The seedlings and micropropagated shoots were removed from the culture vessels and the roots were thoroughly washed in tap water to remove any residual agar. Subsequently, the seedlings were transferred to the uncapped containers that were maintained at room temperature at 25 ±2°C with 70% relative humidity. Finally, these were transferred to the community pots filled with terrestrial compost (soil and leaf litter) in the ratio of 1:1 in the greenhouse.

### Statistical analysis

The cultures were observed regularly under a binocular microscope (Olympus SZX10, Japan) and data recorded accordingly. The experiments followed a completely randomized design. The results were tested by applying Tukey's multiple comparison test ($p \leq 0.05$) in a one-way ANOVA. Statistical analysis was performed using the SPSS (version 16.0) software package (SPSS, Inc., Chicago, IL, USA). The results were expressed as mean ±*SD* of eight replicates. The experiment was repeated twice.

## Results

### In vitro asymbiotic seed germination and seedling development

The seeds showed signs of germination after 8 weeks of culture with swelling of the embryo (Tab. 1, Fig. 2a). The swollen seeds emerged as pale-yellow spherules by rupturing the testa (Fig. 2b). These grew in size while still attached to the testa. During the next 5 weeks, the spherules developed into conical, non-chlorophyllous protocorms. A shoot tip developed at the apical part (Fig. 2c) and roots at the basal part of the protocorm.

Morphogenetic events (i.e., development of spherule, protocorm, differentiation of leaves and roots) were observed to be more advanced in the cultures kept under 12-h photoperiod, as compared with the cultures from darkness. The protocorms developed chlorophyll only after shifting the cultures from dark to 12-h photoperiod. The roots developed after two leaf stage (Fig. 2d). The roots were light brown, long, brittle, hairy all over the surface, and the variegations in the leaves were visible (Fig. 2e). Seedlings developed within 27 weeks.

### Effect of capsule development on seed germination

The percentage of seed germination varied significantly with respect to the stage of the capsule. The seeds from undehisced capsules germinated readily and at a higher frequency than those collected from dehisced, dry, brown capsules (Tab. 2). The seeds collected from the dehisced capsules germinated at reduced frequency of about 20% and after longer time.

### Effect of media and incubation period percentage of seed germination

The highest seed germination of 82.7 % was achieved in BM-1 medium in 12-h photoperiod after 8.1 weeks (Tab. 2, Fig. 3). In BM and KC media, the germination was reduced to 37.4% and 35.7%, respectively. In dark, seed germination was delayed, the germination percentage was reduced and the protocorms were achlorophyllous. The chlorophyll appeared when protocorms were transferred to 12-h photoperiod and developed normal seedlings. Otherwise, if kept under continuous dark the protocorms turned necrotic and died. MM medium failed to support seed germination.

**Tab. 1** In vitro seed germination response of undehisced capsule of *P. venustum* on different media under 12-h photoperiod.

| Media | Germination percentage | Initiation of response (weeks) | Development (weeks) of | | | |
|---|---|---|---|---|---|---|
| | | | protocorm | 1st leaf | 1st root | seedlings |
| BM | 37.40 ±1.00 $^{bd}$ | 12.15 ±0.19 $^{bcd}$ | 17.00 ±0.00 $^{bcd}$ | 21.33 ±0.45 $^{bcd}$ | 27.68 ±0.39 $^{bcd}$ | 33.27 ±0.22 $^{bcd}$ |
| BM-1 | 82.75 ±0.15 $^{acd}$ | 8.15 ±0.17 $^{acd}$ | 13.12 ±0.15 $^{acd}$ | 15.95 ±0.25 $^{acd}$ | 21.88 ±0.25 $^{acd}$ | 26.17 ±0.17 $^{acd}$ |
| KC | 35.75 ±0.05 $^{bd}$ | 12.98 ±0.26 $^{abd}$ | 19.05 ±0.10 $^{abd}$ | 23.42 ±0.41 $^{abd}$ | 29.15 ±0.19 $^{abd}$ | 35.12 ±0.09 $^{abd}$ |
| MM | 0.00 $^{abc}$ | 0.00 $^{abc}$ | 0.00 $^{abc}$ | 0.00 $^{abc}$ | 0.00 $^{abc}$ | 0.00 $^{abc}$ |

BM – terrestrial orchid medium; BM-1 – modified terrestrial orchid medium; MM – Malmgren modified terrestrial orchid medium; KC – Knudson C medium. Values in a column followed by the same letter superscripts are not significantly different at $p \leq 0.05$ according to Tukey's test.

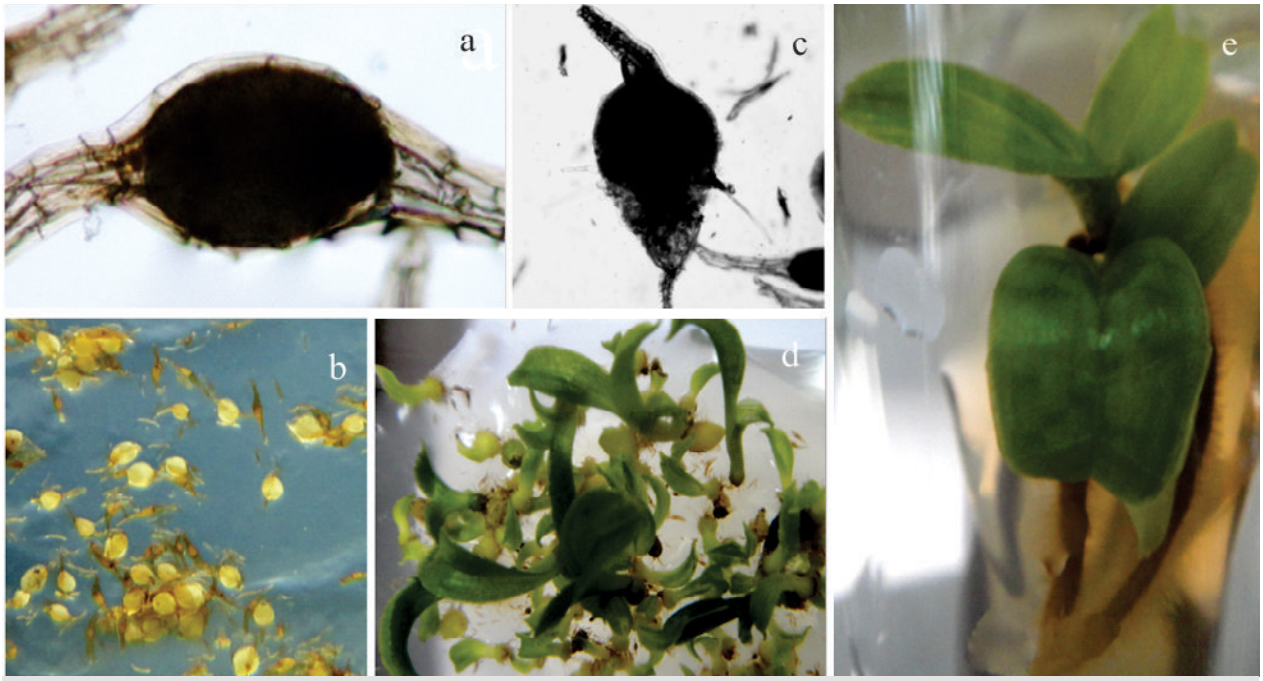


**Fig. 2** In vitro seed germination and seedling development of *Paphiopedilum venustum*. **a** Seeds at the time of inoculation (stage 1). **b** Spherule (stage 2). **c** Appearance of roots at the spherule surface (stage 3). **d** Cultures at protocorm stage (stage 4). **e** Development of healthy shoots (stage 5).

**Tab. 2** The effect of capsule stage on seed germination percentage of *P. venustum* in vitro under 12-h photoperiod in BM-1 medium.

| Capsule stage | Germination % |
|---|---|
| Dehisced | 20.40 ±0.10 $^{a}$ |
| Undehisced | 82.75 ±0.15 $^{b}$ |

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

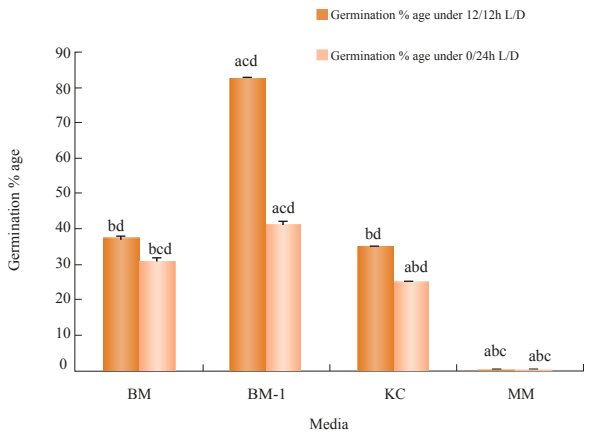


**Fig. 3** Effect of basal media and incubation photoperiod on percentage of seed germination of *Paphiopedilum venustum*.

## Effect of organic growth supplements on shoot multiplication

The treatment with organic supplements considerably increased the number of multiple shoots as compared with control (Fig. 4a,b, Tab. 3). The new shoots emerged as spike-like structures at the base of the shoots (Fig. 4a).

In the medium with 10 g $L^{-1}$ of banana homogenate 2.05 shoots were induced. Higher dose of banana homogenate promoted a healthy growth of seedlings. Phenotypically, the plantlets were robust and lush green with spots of anthocynin pigments visible at the basal portions of the leaves (Fig. 4a). Peptone 1 g $L^{-1}$ in the medium, supported regeneration of 3.05 adventitious shoots per cultured shoots (Fig. 4b). These newly formed shoots rooted and grew well. They were separated carefully from the mother shoot and inoculated into their respective medium for further development. Conversely, potato powder was ineffective in initiating shoot multiplication and was detrimental to the cultures.

## Acclimatization

Before acclimatizing, the seedlings and adventitious plants grew in their respective media for 6 months in the culture flasks. Fortnightly, the seedlings were transferred from full strength PGR-free BM-1 medium to half-strength, one-quarter and one-eighth, and then to plain distilled water gelled with agar, then to water with reduced concentration of agar. Well-rooted plants were planted in the community pots filled with terrestrial compost. During acclimatization, original shoots turned brown and senesced and new leaves and roots developed (Fig. 4c,d). Plants were acclimatized with 80% survival in the greenhouse.

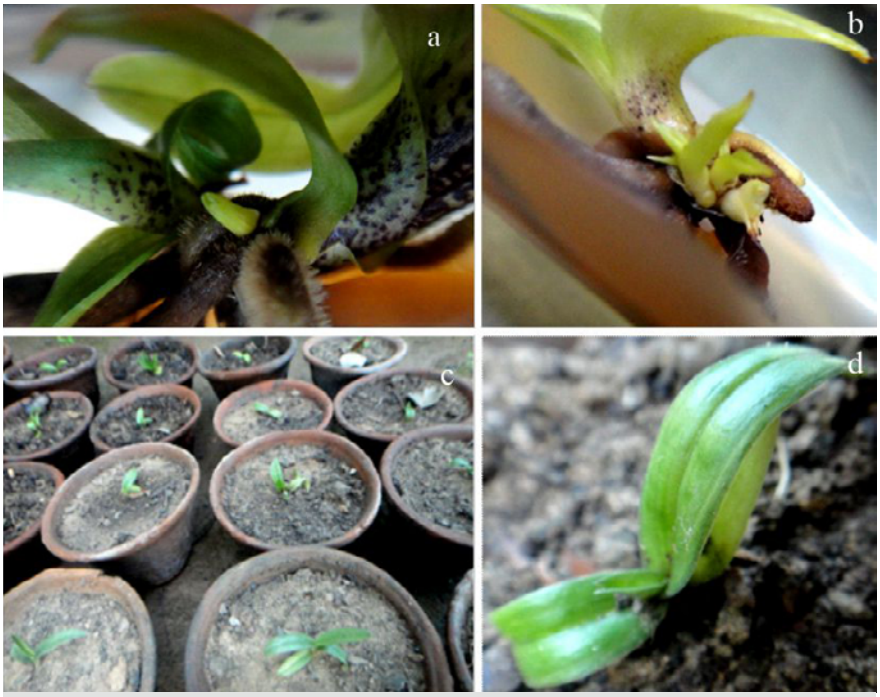


**Fig. 4** Multiplication of the shoots and acclimatization of *Paphiopedilum venustum*. **a** Emergence of new shoots as spike-like structures at the base of the shoots and anthocynin pigments visible at the basal portions of the leaves in bh enriched medium. **b** Maximum shoot induction in peptone enriched medium. **c** Seedlings in community pots in the greenhouse. **d** A healthy seedling growing in a community pot.

**Tab. 3** In vitro multiplication of shoots of *P. venustum* in BM-1 medium with organic growth supplements.

| Organic supplements | Number of shoots induced / shoot | Total number of plantlets/shoot |
|---|---|---|
| BM-1 (control) | 1.00 ±0.00 $^{bdefgh}$ | 2.00 ±0.00 $^{bdefgh}$ |
| –Bh(10) | 2.05 ±0.50 $^{acdefgh}$ | 3.05 ±0.00 $^{acdefgh}$ |
| –Bh(20) | 1.00 ±0.00 $^{bdefgh}$ | 2.00 ±0.00 $^{bdefgh}$ |
| –Bh(30) | 0.00 $^{abce}$ | 1.00 ±0.00 $^{abcegh}$ |
| –P(1) | 3.05 ±0.10 $^{abcdfgh}$ | 4.05 ±0.00 $^{abcdfgh}$ |
| –P(2) | 0.00 $^{abce}$ | 1.00 ±0.00 $^{abcegh}$ |
| –Pp(5) | 0.00 $^{abce}$ | 0.00 $^{abcdef}$ |
| –Pp(10) | 0.00 $^{abce}$ | 0.00 $^{abcdef}$ |

Bh – banana homogenate; P – peptone; Pp – potato powder. All concentration = g $L^{-1}$ ($wv^{-1}$). Values in a column followed by the same letter superscripts are not significantly different at $p \leq 0.05$ according to Tukey's test.

## Discussion

Application of in vitro techniques helps in the conservation of the germplasm of threatened species. In orchids, asymbiotic seed germination on a defined medium is advantageous as it saves the time-gap between pollination and seed sowing and may be applied in rescuing hybrid embryos. Furthermore, tissue culture can be used to clone apomictic taxa, propagate desired genotypes and is used in genetic transformation studies [26,27].

In our experiment, the seed germination percentage was significantly influenced by maturity level of the capsule, type of medium used, and light condition. The seeds, collected from undehisced capsules germinated at a higher frequency than those from dehisced brown (mature) capsules. This is most likely due to the former being metabolically active. The earliest stage at which the immature seeds are able to germinate could not be examined in the present species. Earlier studies in *Dendrobium nobile* and *Phalaenopsis amabilis* var. *formosa* showed positive correlation between the germination abilities of immature seeds with their internal organization such as discontinuous cuticle layer enveloping the embryo, presence of gaps which are caused by the cellular degeneration of the inner integument, and the absence of secondary wall thickenings in the outer integument. All these features of the seed integuments contribute to maximizing the percentage of germination of immature seeds [28,29].

Literature survey indicates a correlation between maturity related progressive decline of germination percentage to the biochemical changes, which induce dormancy in the seeds, by accumulating inhibitory substances leading to the rapid loss of viability [18,29–34]. As the embryo matures, it dehydrates due to the development of hydrophobic carapace sheath and lignification of the cell walls of testa layer. The level of endogenous abscisic acid also increases which is also responsible for decline in the germination percentage [35].

In our study, seed germination of *P. venustum* was accomplished in most of the media, regardless of the duration of light treatment, similar to those reported earlier in *Paphiopedilum sukhakulii* [36], *Calopogon tuberosus* [37], *Bletia purpurea* [38], *Phragmipedium humboldti*, *P. longifolium*, and *P. pearcei* [39]. In our cultures, a treatment with 12-h photoperiod favored early onset of germination with better germination percentage as compared with complete darkness. In many terrestrial orchids, light is a limiting factor for seed germination [17,34,40] and the photoperiod responses in the germination of terrestrial orchid seeds are species specific [41].

In the present work, the highest seed germination frequency was achieved in BM-1 followed by BM, and KC medium. There was no germination observed in MM medium. BM-1 and BM medium contained more of organic form of nitrogen. Inorganic form of nitrogen of KC appeared to have reduced the germination percentage possibly

due to a lower activation of nitrate reductase enzyme as it was also suggested by others [18,42,43]. Our results corroborate earlier reports on *Paphiopedilum* species in which organic compounds favored better seed germination [38,44].

The chlorophyll synthesis was observed to be a post-protocorm phenomenon in our cultures. According to Stoutamire [30] non-chlorophyllous protocorms characterize the species growing in well-drained soils. *Paphiopedilum venustum* inhabits ground soils, and non-chlorophyllous protocorms seems to be a genetic attribute. Chlorophyll production was faster in cultures under 12-h photoperiod than those under dark in which it appeared only when the cultures were shifted to 12-h photoperiod as observed in other orchid species [7]. In a natural habitat, *P. venustum* prefers to grow in light and moderate shady environment. Under in vitro conditions, the species showed maximum germination in light, which most likely simulated similar environmental conditions.

The presence of organic supplements in the culture medium contributes towards the development of a simple and economical plant growth medium and limits the use of exogenous PGRs. This reduces the frequency of occurrence of undesired somaclonal variations [45]. Growth supplements are able to influence in vitro regeneration, multiplication of protocorm-like body(ies) and growth of the orchid seedlings, their efficacy has been tested in a few *Paphiopedilum* species and hybrids such as *Paphiopedilum phillippinese* × *Paphiopedilum* 'Susan Booth' [46], *Paphiopedilum rothschildianum* [8].

In earlier studies, peptone was reported to have induced multiple shoots in *Paphiopedilum* species and its hybrids such as *Paphiopedilum* 'Alma Gavert', generating an average of three adventitious (de novo) shoots from a single shoot [47], in *Paphiopedilum rothschildianum*, an average of 2.8 shoots [17], and multiple shoots were initiated in *Cymbidium macrorhizon* and other *Cymbidium* species [48]. In our experiment, peptone successfully induced multiple shoots. A maximum number of three shoots were generated per shoot and a total of four plantlets were obtained. The beneficial effect of peptone (a water-soluble protein hydrolysate) is due to its high content of amino acids, low molecular weight proteins, and vitamins that promote growth of the cultures [49] as was earlier indicated in *Cymbidium macrorhizon* and *Cymbidium species* [50]. On the other hand, bh-supplemented medium generated a maximum of two adventitious shoots per culture and promoted development of healthy plantlets which could be attributed to medium's higher sucrose concentration as has been suggested earlier in *Dendrobium* species [49]. The promoting effect of bh in supporting proliferations of secondary protocorm-like bodies (PLBs) in *Paphiopedilum rothschildianum* [16], healthy growth of plantlets in *Dendrobium chrysotoxum* and *Cymbidium pendulum* [51] have been reported.

Potato powder proved to be detrimental to the cultures. Our results are in accordance with earlier findings in *Phalaenopsis gigantea* where potato homogenate hindered the growth index of protocorms which turned pale and stunted [52]. The detrimental effect of potato powder is attributed to causing a decrease in the water osmotic potential of a medium from hydrolysis of starch at a high temperature during autoclaving [7]. The seedlings were acclimatized in the greenhouse and developed new leaves and roots as reported in *Bletia purpurea* seedlings [38].

## Conclusions

This study reports a simple protocol for the propagation of *P. venustum* through in vitro asymbiotic seed germination and the induction of multiple adventitious shoots without the use of PGRs. Under 12-h photoperiod, BM-1 supported high seed germination and seedling development. A 12-h photoperiod was necessary for the differentiation of protocorms. Peptone (1 g $L^{-1}$) was highly beneficial for multiplication of the shoots. We further focus on exploring phyto-chemical constituents of *P. venustum* and restoring the species in its natural habitat.

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