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# Research Article
# *In vitro* Immature Embryo Culture and Propagation of *Paphiopedilum villosum* Var. *Boxallii* (Rchb. f.) Pfitzer

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## Abstract

**Background and Objective:** *Paphiopedilum* Pfitzer (Orchidaceae) is among the most popular orchid genera that have been successfully commercialized as potted plants and cut flowers due to the peculiarity of its flowers. *Paphiopedilum villosum* var. *boxallii* is a rare and vulnerable horticultural important species. The aim of this study was to develop the *in vitro* propagation protocol from immature seeds. **Materials and Methods:** Seeds of various developmental age (150-300 days after pollination, DAP) of *P. villosum* var. *boxallii* were cultured on different nutrient media supplemented with different organic carbon source, coconut water, activated charcoal, NAA and BA at different combination. **Results:** Seeds from ~240 DAP old pods registered 60% germination on MS medium containing sucrose (3%, w/v), NAA and BA (2 and 6 μM, respectively in combination) within 59 days under diffused light (20 μmol $m^{-2}$ $sec^{-1}$). Incorporation of coconut water did not support seed germination but supported early differentiation to protocorm-like bodies (PLBs). Germinated seeds converted to PLBs and differentiated to plantlets. The PLBs converted to rooted plantlets full strength MS medium fortified with sucrose (3%), NAA+BA (2+6 μM in combination) where as many as 7 shoot developed/explants per sub-culture cycle. The rooted plantlets were hardened by culturing on 1/10th MS medium containing sucrose (1%) and hardening matrix (sand, decaying organic matter, brick pieces, charcoal and dried cow dung at 1:1:1:1:1 ratio). The hardened plants were transferred to potting mix along with the hardening matrix where ~65% transplants survived in the green house condition. **Conclusion:** This study opens up an effective alternate route of propagation of this vulnerable species *Paphiopedilum villosum* var. *boxallii* (Rchb. f.) Pfitzer.

Key words: Asymbiotic seed germination, floricultural orchid, *in vitro* propagation, *Paphiopedilum villosum* var. *boxallii*, vulnerable species

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

Genus *Paphiopedilum* Pfitzer (Orchidaceae), commonly known as Lady's slipper orchid have captured the interest of orchid growers and hobbyists because of their beautiful flowers and is one of the rare group of orchids[1]. Over collection, losses of habitat and environmental disruption have led to the rapid decline of their natural populations. Hence, the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) have categorized *Paphiopedilum* species under Appendix I which prohibits the trade of this genus. Nine species of *Paphiopedilum* are recorded from India and is protected under Wildlife Protection Act of India, 1972, Schedule VI. *Paphiopedilum villosum* var. *boxallii* is rare and has only been recorded from China (Yingjiang County, Yunnan), Myanmar and North eastern region of India (Fig. 1a). The species has been categorized as Vulnerable (VU) by the IUCN Red List of threatened species[2]. Population of the species in its natural habitats is under threat due to over collection, habitat destruction and other anthropogenic activities. *Paphiopedilum* species and their hybrids are the only commercially grown orchids[3,4] but there are very few protocols available for production of clones. Conventionally propagation is done by division of axillary buds from the mother plant which is time consuming and not commercially viable.

*In vitro* propagation technique is a viable alternative for large scale production of plantlets of commercially viable species and conservation of rare and threatened species. For commercial purpose it desires genetically uniform planting materials which are produced from somatic cells/tissues. *Paphiopedilum* species and their hybrids are the only commercially grown orchids that are not cloned since explants from mature plants of *Paphiopedilum* species are recalcitrant to shoot induction and plant regeneration[5]. Further, it is difficult to establish *in vitro* culture of *Paphiopedilum* from explants of *in vivo* source/mature plants due to endogenous bacterial contamination as surface sterilization steps inefficient[6]. Due to this reason most of the *Paphiopedilum* species are propagated from seed culture. Though this species an economically important species, there is no report available for production of planting material and conservation. The present study was aimed at development of protocol for asymbiotic seed germination and production of planting materials for conservation of *Paphiopedilum villosum* var. *boxallii*.

## MATERIALS AND METHODS

**Plant material:** Green pods of different growth age (120-300 days after pollination, DAP) of *Paphiopedilum villosum* var. *boxallii* were harvested at 30 days interval (Table 1) from the plants maintained in the orchidarium of Department of Botany, Nagaland University, India (Fig. 1b, 1-4). Present study was undertaken during August, 2015 and completed in March, 2018. Collected pods were surface cleansed by scrubbing with soft brush and diluted Laboratory detergent (Labolene, 1:100 v/v) and washed repeatedly in running tap water. Subsequently surface cleansed pods were sterilized with aqueous solution of mercuric chloride (0.3%, w/v) for 5 min followed by rinsing with sterilized water. The pods were dipped in ethanol (70%, v/v) and flamed for ~5-7 sec before scoping out the seeds for culture.

**Nutrient media:** Different nutrient media viz., Murashige and Skoog[7] (MS), Gamborg *et al.*[8] or (B5), Mitra *et al.*[9], Knudson[10] 'C' and SH[11] were used in conjunct with various supplements for asymbiotic *in vitro* germination of seeds. Nutrient media were fortified differently with organic carbon sources such as dextrose, glucose and sucrose (0-4%, w/v), plant growth regulators (PGRs) viz., α-naphthalene acetic acid (NAA, 0-6 µM), benzyl adenine (BA, 0-10 µM) either singly or in combination, tender coconut water (CW) (0-25%, v/v) and activated charcoal (AC) (0-0.5%, w/v) and 0.8%, w/v agar was added as gelling agent. The pH of the media was adjusted at 5.6 using 0.1 N NaOH and 0.1 N HCl and ~12 mL medium was dispensed in each borosilicate test tube (size 25×150 mm) and media was autoclaved at 121°C for 20 min at a pressure of 1.05 kg $cm^{-2}$.

### Initiation of culture

**Asymbiotic seed germination:** Seeds were scoped out from the sterilized pods and cultured on media as stated above. Cultures were maintained at 25±2°C under 40 µmol $m^{-2}$ $sec^{-1}$ illumination, diffused light (20 µmol $m^{-2}$ $sec^{-1}$) provided by white fluorescent lamps with 12/12 h photoperiod and in the dark. The cultured seeds were monitored at regular interval for germination and initial germination proceedings were observed under light microscope. For the purpose, a part of the cultured seeds were scoped out randomly and stained on microscopic slide with acetocarmine (2%, w/v, prepared following Darlington and La Cour[12]). Germinated embryos formed PLBs

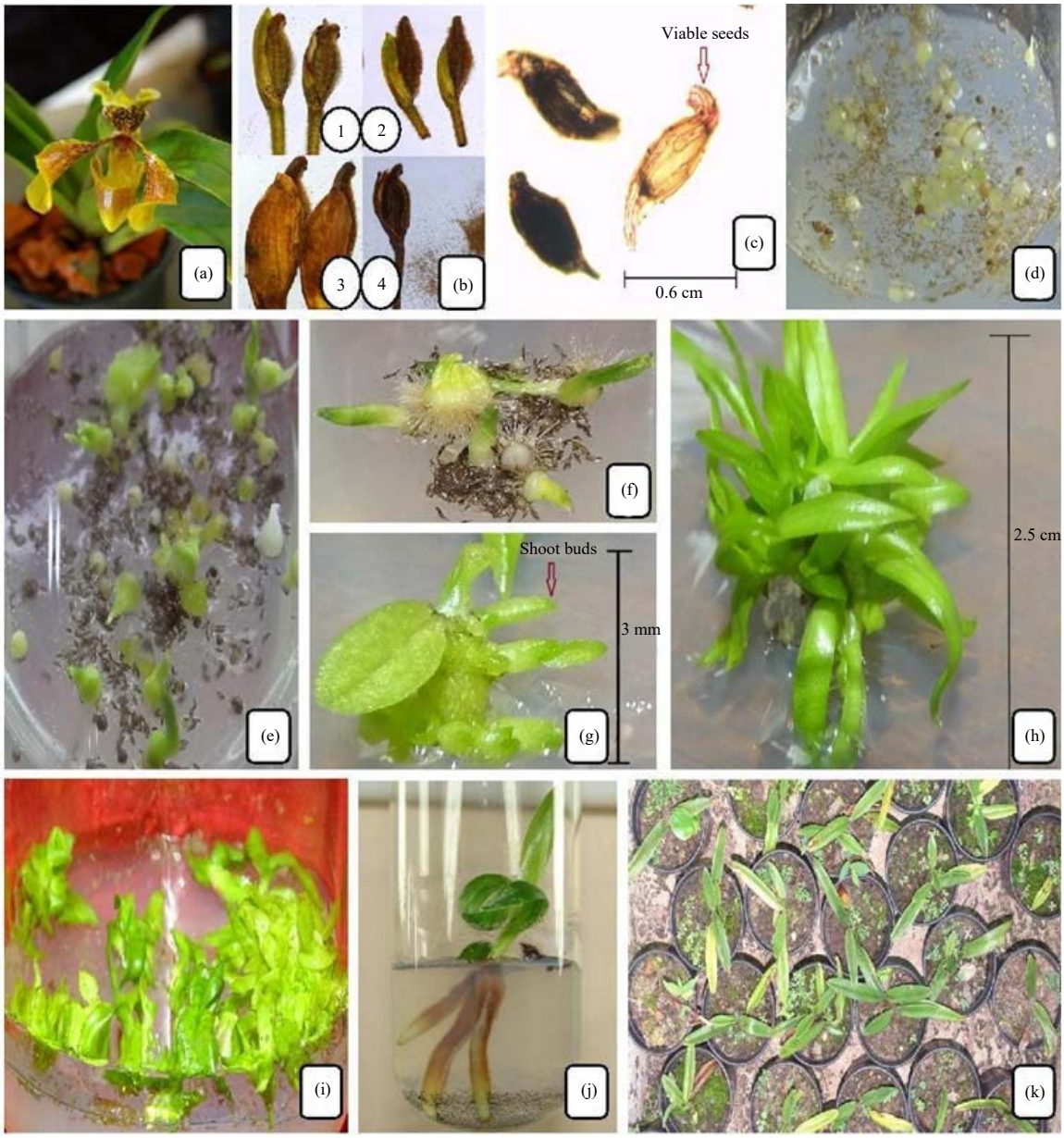


Fig. 1(a-k): Various Stages of micropropagation of *Paphiopedilum villosum* var. *boxallii*, (a) Flowering plant, (b) Different aged seed pods/capsules [(1) 120 DAP old, (2) 180 DAP pods, (3) 240 DAP pod and (4) 300 DAP pods)], (c) Viable seeds viewed under a stereo microscope, (d) Nodular swelling of the embryos and separation of testa showing the first sign of germination, (e) Healthy PLBs formed from seeds/embryos from older aged pods (>210 DAP), (f) Micro plants with first leaf initials, (g) A maximum of ~ 7 shoot buds developed from foliar nodal explants at a combination of NAA and BA in the ratio 2:6 µM (v/v), (h) Multiple shoot growth from shoot buds developed from foliar nodal explants, (i) Mass multiplication and regeneration with healthy leaves and root, (j) Healthy single shoot and root growth recorded at NAA constant 2 µM and BA at 8 and 10 µM (v/v) and (k) Hardened plants with healthy growth in the greenhouse

on the same initiation medium and released the first set of leaves. Cultures were sub-cultured on optimum germination condition for another 2 passages at 4-5 weeks interval for further differentiation. Cultures were monitored at weekly interval for growth of plants, plant differentiation and generation of root.

Table 1: Effect of green pod age on *in vitro* germination of *P. villosum* var. *boxallii* seeds

| Green pod age (DAP) | Time (days)* 1st sign nodulation | PLBs formation | 1st leaf initials | Germination (%) (±SE)# |
|---|---|---|---|---|
| 120 | - | - | - | - |
| 150 | 18 | - | - | - |
| 180 | 24 | 44 | 76 | 12.1 (1.5) |
| 210 | 39 | 54 | 80 | 25.2 (1.5) |
| 240 | 42 | 59 | 82 | 60.0 (1.5) |
| 270 | 45 | 83 | 112 | 42.2 (2.5) |
| 300 | 45 | 89 | 122 | 44.2 (2.5) |

*Cultured on MS medium with sucrose (3%), NAA and BA (2 and 6 µM respectively in combination), #Standard error from mean

**Shoot proliferation, plantlets regeneration and rooting of regenerate:** The resulted PLBs, young plantlets obtained from cultured seeds were transferred on 3 different nutrient media different nutrient media (Knudson[10] 'C', Mitra *et al.*[9] and MS at different strengths i.e., 1/4th, 1/2, 3/4th and full strength) along with other adjuncts such as organic carbon source (dextrose, glucose and sucrose, 0-4%) and PGRs for regeneration of plantlets and culture proliferation. Four different PGRs viz., NAA, IAA, BA and KN (0-8 µM either singly or in combination) were tested for culture proliferation and plant regeneration. Cultures were maintained for 8-10 weeks under normal laboratory condition.

**Hardening and transplanting of regenerates:** For primary hardening, regenerated plantlets (~5 cm) with well expanded leaves, healthy shoot and roots were selected and transferred in culture vials containing different low cost substrata/hardening mix. The hardening matrix was made by mixing of chopped coconut husk, brick pieces, charcoal chunks, sand, decaying organic matter, dried cow dung at different combinations as follows:

- Chopped coconut husk
- Brick pieces+charcoal chunks+chopped coconut husk at 1:1:1 ratio
- Sand+decaying organic matter at 1:1 ratio
- Sand+decaying organic matter+brick pieces+charcoal chunks+dried cow dung powder at 1:1:1:1:1 ratio
- Wooden pieces. Substrata were soaked in laboratory detergent for ~1 h and rinsed several times with water before sun drying for 6-7 h

About 50-60 g of substrata was transferred to each culture vial (size 450 mL) and autoclaved at 121°C for 30 min at pressure of 1.05 kg $cm^{-2}$. About 20 mL 1/10th strength MS liquid medium (containing sucrose, 1% but devoid of any PGRs) was dispensed in each culture vial containing low cost substrata and autoclaved at 121°C for 20 min. The well rooted plantlets were transferred in the culture vials and maintained in normal Laboratory condition for 7-8 weeks. The primary hardened plants were then transferred to community potting mix composed of same substrates used for hardening, covered with perforated poly bags and maintained in the poly house ca. 75% shade for 6-8 weeks. The transplants were fed water and 1/10th MS solution weekly. The well established transplants were transferred to wild after 2 months and monitored for survival performance.

**Experimental design and statistical analysis:** Completely randomized experimental design was followed. For each treatment, 10 culture vials were maintained and repeated thrice. *In vitro* response was calculated based on percentage of explants responded and number of propagules formed in the culture and data was expressed as a mean of replicates ±standard error. Data was further analyzed by one way ANOVA using the general Linear Model procedure in SAS Statistical Package (SAS Ins.) and standard deviation from mean was worked out and compared using least significant difference (LSD) test at $p = 0.05$.

## RESULTS

**Initiation of cultures:** First sign of germination was observed as nodular swelling of embryos followed by rapture of testa and enlargement of embryo, subsequently formation of top-shaped Protocorm-like bodies (PLBs) (Fig. 1c, d). Enlargement of embryos and emergence of the testa was considered as criteria for seed germination. *In vitro* asymbiotic seed germination of *P. villosum* var. *baxallii* was influenced by many factors viz., developmental age of the seeds/green pods, nutrient medium, quality and quantity of PGRs, organic carbon etc.

**Developmental age of seeds:** Different orchid species respond differently on various basal medium and adjuvant and hence homogeneity of a single protocol is not expected

across all orchid species. In the present study too seed pod age was a crucial factor for successful asymbiotic germination. Green pod age of 120-300 DAP were used for culture initiation at an interval of 30 days (Table 1). Younger seeds (~120 DAP) did not show any sign of germination, while seeds from >270 DAP old pods exhibited delayed germination with very low germination. Seeds collected from burst out seed pods (Fig. 1b, 4) (~300 DAP) were also collected and used for asymbiotic germination but registered delayed germination (~6 months) accompanied by poor germination and high contamination. For calculating seed viability, the scoped out seeds from capsule were stained in acetocarmine (2%, w/v) for 10 min and viewed under light microscope. The stained seeds were considered as viable seeds (Fig. 1c). Nodular swelling of the embryos and separation of testa showing the first sign of germination was recorded at early as 18 days of culture (Table 1) but no/very few PLBs were formed from seeds/embryos of very young aged pods (<150 DAP). Healthy PLBs (Fig. 1d, e) were formed only in seeds from older aged pods (>210 DAP) and subsequently healthy PLBs and plantlets with leaf initials (Fig. 1f). Under the given conditions, 59% germination was achieved from seeds of 240 DAP while the older seeds prolonged the germination process (Table 1).

**Effect of nutrient medium:** Of the 5 different nutrient media studied in the present study, MS medium supported optimum germination (60%) followed by Knudson[10] 'C' (40.2%) and Mitra *et al.*[9] (35.2%) while, Gamborg *et al.*[8] (B5) and SH medium supported very low germination (Table 2). Gamborg *et al.*[8] ($B_5$) and Knudson[10] 'C' media supported slight swelling of the embryos but did not convert to PLBs, while, on Mitra *et al.*[9] medium nodular swelling of embryos as well as formation of PLBs were observed but the PLBs failed to differentiate into plantlets.

**Effect of organic carbon sources, CW and AC on *in vitro* embryo culture:** For a symbiotic germination of seeds, varying concentrations of different carbon sources i.e., dextrose, glucose and sucrose (0-4%, w/v) were incorporated. Exclusion of organic carbon in the nutrient medium recorded only nodular swellings of seeds. In the present study, 3 different organic carbon sources (dextrose, glucose and sucrose) were used at varying strengths (1-4%) in MS medium (Table 3). Seeds cultured on organic carbon free medium supported only nodular swelling of embryos and rupturing of the test a without formation of PLBs. Incorporation of at least one organic carbon source was perquisite for successful

Table 2: Effect of nutrient media on non-symbiotic seed germination of *P. villosum* var. *boxallii*

| Nutrient medium | Germination time (days) | Germination (%) (±SE)* |
|---|---|---|
| Gamborg *et al.*[8] ($B_5$) | 130 | 20.1 (1.5) |
| Knudson[10] 'C' | 90 | 40.2 (1.5) |
| Mitra *et al.*[9] | 80 | 35.2 (1.5) |
| MS | 59 | 60.0 (1.5) |
| SH | 130 | 25.2 (2.0) |

*Standard error from mean, data represents mean of 3 replicates, about 240 DAP old seeds cultured on medium fortified with sucrose (3%), NAA and BA (2 and 6 µM, respectively in combination)

Table 3: Effect of quality and quantity on nutrient medium additives organic carbon source, coconut water (CW) and activated charcoal (AC) on *in vitro* embryo culture of *P. villosum* var. *boxallii*

| Organic carbon source and concentration | Days for germination | Germination (%) (±SE)# | CW concentration (%) | Days for germination | Germination (%) (±SE)# |
|---|---|---|---|---|---|
| Control | 92 | 22.2 (1.5) | 0 | 59 | 60.0 (1.5) |
| Dextrose | | | 5 | 53 | 54.2 (1.50 |
| 1 | 76 | 36.2 (1.5) | 10 | 52 | 53.3 (1.5) |
| 2 | 74 | 22.3 (1.5) | 15 | 58 | 56.4 (1.5) |
| 3 | 76 | 24.1 (1.5) | 20 | 62 | 55.5 (2.5) |
| 4 | 88 | 20.2 (2.0) | 25 | 64 | 54.2 (2.5) |
| **Glucose** | | | | | |
| **AC concentration (%, w/v)** | | | | | |
| 1 | 75 | 28.1 (1.5) | 0.05 | 62 | 52.1 (1.5) |
| 2 | 78 | 32.1 (1.6) | 0.10 | 68 | 50.4 (1.5) |
| 3 | 72 | 30.4 (1.2) | 0.20 | 73 | 38.2 (1.2) |
| 4 | 79 | 32.2 (2.0) | 0.30 | 108 | 04.1 (0.5) |
| **Sucrose** | | | | | |
| 1 | 65 | 38.3 (1.5) | 0.50 | 112 | 03.1 (0.3) |
| 2 | 68 | 42.3 (1.5) | | | |
| 3 | 59 | 60.0 (1.5) | | | |
| 4 | 82 | 45.2 (2.0) | | | |

#Standard error from mean, data represents mean of 3 replicates, seeds of ~240 DAP old cultured on MS medium containing NAA and BA (2 and 6 µM respectively in combination)

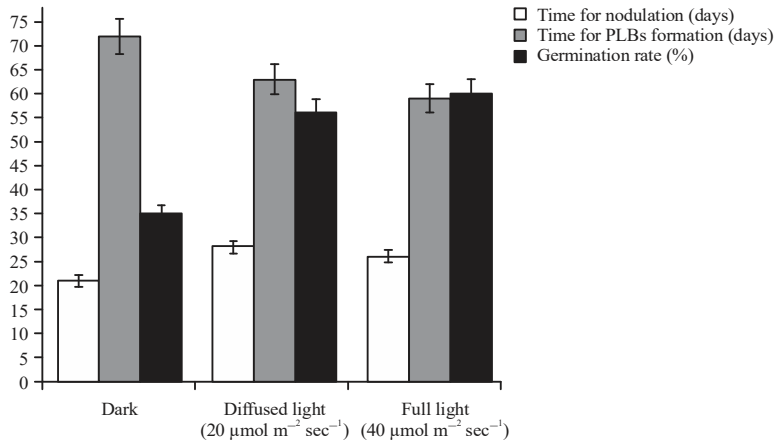


Fig. 2: Effect of light on *in vitro* culture of immature embryos of *P. villosum* var. *boxallii*

*in vitro* culture of seeds. Different levels of organic carbon sources exhibited different effect on seed germination. The immature seeds responded more or less identically on dextrose and glucose enriched medium across the concentrations (Table 3) while, sucrose fortified medium supported faster germination (within 59 days) and subsequent differentiation to PLBs. At lower and higher concentrations (1, 2 and 4%) germination rates was significantly lower (38.3, 42.3 and 45.2% respectively). Under the given conditions, optimum germination was achieved on MS medium enriched with 3% sucrose where 60% seed registered germination followed by PLBs formation (Table 3, Fig. 1d, e).

Besides organic carbon, role of CW on asymbiotic *in vitro* seed germination and PLBs development of *P. villosum* var. *boxallii* was studied. In the present investigation, use of CW had little or no effect on seed germination compared to CW control medium. However had a promontory effect on early differentiation into PLBs at lower concentrations ($\leq$15%) (Table 3). While, study AC (0.05-0.1%) supported formation of healthy PLBs but with increase in concentration (0.2-0.5%) it had negative and embryos tend to form nodular swelling only (Table 3).

**Effects of light intensity on seed germination:** Cultured seeds were maintained in 3 different light conditions including dark. Nodulation of seeds was observed after 21 days in the dark. Though initial sign of germination was faster in the dark but germination rate was lower compared to embryos maintained under diffused light (20 µmol $m^{-2}$ $sec^{-1}$). Optimum germination of 60% was recorded under diffused light (20 µmol $m^{-2}$ $sec^{-1}$) (Fig. 2).

**Effects of PGRs:** Of the two PGRs tested at different concentrations, BA slightly enjoyed upper hand over NAA. On NAA fortified medium though swelling of seeds observed, failed to form healthy PLBs as exhibited on BA fortified medium. Medium fortified with NAA at low concentration (2 and 4 µM) supported few PLBs formation but at higher concentration (6 µM) supported only swelling of embryos after 90 days of culture. Conversely, BA at lower concentrations (2-4 µM) registered only swelling of seeds without formation of PLBs but the trend reversed at higher concentrations (6-10 µM) where in most of the cases PLBs resulted but, failed to differentiate into plantlets. Synergistic treatment of NAA and BA improved the germination pattern significantly. Optimum germination (60%) was achieved on medium fortified with NAA+BA (2.0+6.0 µM in combination) accompanied with PLBs formation with first leaf initials (Table 4, Fig. 1f).

**Regeneration and mass multiplication:** The resultant PLBs were maintained for 8-10 week for further differentiation and proliferation on optimum initiation conditions. The advanced stage PLBs and shoots transferred on regeneration medium for plantlet regeneration and culture proliferation (Fig. 1g). Plant regeneration and culture proliferation were affected by various factors like quality and quantity of nutrient medium, organic carbon source and quantity, quality and quantity of PGR etc.

**Effect of basal medium:** Of the nutrient media (Knudson[10] 'C', Mitra *et al.*[9] and MS) at different strengths tested for plant regeneration and culture proliferation, MS medium found to be ideal. An average of 7 shoot buds developed per explant per subculture after 21 days of transfer on multiplication medium. Subsequently, the shoot buds developed leaves with roots and differentiated into rooted plantlets (Fig. 1 h-j). Other 2 media viz., Knudson[10] 'C' and Mitra *et al.*[9] were

Table 4: Effect of quality and quantity of PGRs on non-symbiotic seed germination of *P. villosum* var. *boxallii*

| PGRs concentration (μM) | | | | |
|---|---|---|---|---|
| NAA | BA | Germination time (Days) | Germination (%) (±SE) * | Type of response |
| 0 | 0 | 64 | 40.2 (1.5) | Nodular swelling of the embryos |
| 2 | 0 | 62 | 42.3 (1.5) | Nodular swelling of the embryos |
| 4 | 0 | 62 | 40.1 (1.5) | Nodular swelling of the embryos |
| 6 | 0 | 60 | 35.2 (1.5) | Nodular swelling of the embryos |
| 8 | 0 | 62 | 38.3 (1.5) | Nodular swelling of the embryos |
| 10 | 0 | 62 | 27.5 (2.0) | Nodular swelling of the embryos |
| 0 | 2 | 92 | 32.3 (2.0) | Nodular swelling of the embryos, No PLBs formed |
| 0 | 4 | 94 | 34.2 (2.0) | Nodular swelling of the embryos, No PLBs formed |
| 0 | 6 | 76 | 42.3 (1.5) | Green PLBs formed with first leaf initials |
| 0 | 8 | 76 | 43.4 (1.5) | Green PLBs formed with first leaf initials |
| 0 | 10 | 78 | 38.5 (1.5) | Green PLBs formed with first leaf initials |
| 2 | 2 | 74 | 40.2 (1.5) | Delayed germination, PLBs formed subsequently degenerated |
| 2 | 4 | 63 | 52.3 (2.0) | Healthy green PLBs formed |
| 2 | 6 | 59 | 60.0 (1.5) | Most of the geminated embryos converted into healthy PLBs and released first leaflets |
| 2 | 8 | 62 | 54.2 (1.5) | Most of the geminated embryos converted into healthy PLBs and released first leaflets |
| 2 | 10 | 64 | 48.1 (1.5) | Most of the geminated embryos converted into healthy PLBs |
| 4 | 2 | 72 | 32.1 (1.5) | Nodular swelling of embryo, delayed germination |
| 4 | 4 | 76 | 28.2 (1.5) | Nodular swelling of embryo, No PLBs formed |
| 4 | 6 | 82 | 30.1 (2.0) | Nodular swelling of embryo, No PLBs formed |
| 4 | 8 | 65 | 43.2 (1.5) | Green PLBs formed |
| 4 | 10 | 64 | 42.2 (1.5) | Germinated embryos converted into healthy green PLBs |
| 6 | 2 | 68 | 40.3 (2.0) | Nodular swelling of the embryos, no PLBs formed |
| 6 | 4 | 70 | 42.2 (2.0) | Nodular swelling of the embryos, no PLBs formed |
| 6 | 6 | 70 | 45.0 (2.0) | Nodular swelling of the embryos, no PLBs formed |
| 6 | 8 | 71 | 44.5 (2.5) | Nodular swelling of the embryos, no PLBs formed |
| 6 | 10 | 71 | 43.2 (1.5) | Nodular swelling of the embryos, no PLBs formed |

*Standard error from mean, data represents mean of 3 replicates, immature seeds of ~240 DAP old cultured on MS medium containing sucrose (3%)

Table 5: Effect of nutrient media and strengths on regeneration of plantlets of *Paphiopedilum villosum* var. *boxallii*

| Basal medium and strength | No. of shoot buds formed/explants* | Time (days) | | Average plant height (cm) (±SE) * |
|---|---|---|---|---|
| | | 1st leaf | 1st root | |
| **Knudson[10] 'C'** | | | | |
| 1/4th | 1 | 67 | - | 0.9 (0.2) |
| 1/2 | 1 | 67 | - | 0.9 (0.3) |
| 3/4th | 1 | 54 | - | 1.2 (0.2) |
| Full | 1 | 32 | - | 1.7 (0.1) |
| **Mitra *et al.*[9]** | | | | |
| 1/4th | - | 31 | 60 | 1.2 (0.3) |
| 1/2 | 2 | 31 | 54 | 1.7 (0.3) |
| 3/4th | 3 | 27 | 49 | 2.2 (0.2) |
| Full | 3 | 25 | 56 | 2.7 (0.2) |
| **MS** | | | | |
| 1/4th | - | | | 1.0 (0.1) |
| 1/2 | 4 | | | 2.5 (0.2) |
| 3/4th | 5 | | | 4.1 (0.2) |
| Full | 7 | 21 | 32 | 4.0 (0.2) |

*Standard error from mean, data represents mean of 3 replicates, on media containing sucrose 3% (w/v), NAA+BA (2+6 μM) in combination

found to be not ideal for culture proliferation of *Paphiopedilum villosum* var. *boxallii* (Table 5). Further, of the different strengths of nutrient medium studied, in general different strengths of MS medium was found to be superior over their counterparts (Table 5). Lower strengths (1/4th, 1/2 strengths) recorded stunted plantlet growth/slower growth with fewer shoot buds formed per subculture suggesting that lower nutrient composition had an adverse effect on plant regeneration.

**Effects of different organic carbon:** In the present study effect of 3 organic carbon sources (dextrose, glucose and sucrose, 0-4%) was investigated for culture proliferation. Sucrose fortified medium supported optimum differentiation

and culture proliferation of *P. villosum* var. *boxallii* while, on dextrose enriched medium recorded stunted root growth though shoot growth was satisfactory (data not presented). Glucose enriched medium supported moderate differentiation up to a concentration of 2% but at higher concentration (3-4%) abnormal shoot growth was recorded.

**Effect of PGRs:** The effect of different PGRs on regeneration and mass multiplication were studied and results recorded therein:

Four different PGRs viz., NAA, IAA, BA and KN were used in the present investigation for plant regeneration culture proliferation. Use of NAA and BA in combination found to be beneficial for regeneration and mass multiplication. At concentration of 2 and 6 µM of NAA and BA respectively in combination as many as 7 shoot buds wherein the first leaf was observed after 21 days of culture while rooting observed after 32 days of culture (Table 6). Nutrient medium fortified with NAA alone did not support PLBs formation while, BA alone supported multiple shoots with elongated leaves accompanied by poor root growth. IAA alone supported formation of no/very few PLBs but with time the explants and PLBs degenerated. IAA in combination with KN across studied concentrations had similar inhibitory effect on seedling growth. In the present study optimum results for healthy root and shoot growth without formation of multiple shoots were observed at combination of NAA (4 µM) and BA (8 µM) (Fig. 1j).

**Hardening and field trials of the regenerates:** Before field trials, hardening of *in vitro* raised plantlets is necessary for better survival and successful establishment of the regenerates. The survival percentage is hence, finally determined by the hardening of the plantlets. Usually, the tissue culture raised plants are hardened by transferring on a low nutrient medium having low organic carbon sources and maintained at increasing light intensity. About 5 cm long rooted plantlets were hardened by maintaining on different substrata at different combinations. The hardened *in vitro* plantlets were then transferred in community pots containing different potting mix combinations (Table 7). Transplants were

Table 6: Effect of different PGRs on plant regeneration and culture proliferation of *Paphiopedilum villosum* var. *boxallii*

| PGRs concentration (µM)* | | | | | Time (days) | |
|---|---|---|---|---|---|---|
| NAA | IAA | BA | KN | No. of shoot buds, PLBs formed/explants | 1st leaf | 1st root |
| 0 | 0 | 0 | 0 | 1 | 68 | 79 |
| 2 | - | - | - | - | - | - |
| 4 | - | - | - | - | - | - |
| 6 | - | - | - | - | 38 | - |
| 8 | - | - | - | - | - | - |
| - | 2 | - | - | - | - | - |
| - | 4 | - | - | - | - | - |
| - | 6 | - | - | 1 | 32 | 43 |
| - | 8 | - | - | - | - | - |
| - | - | 2 | - | - | - | - |
| - | - | 4 | - | 3 | 28 | - |
| - | - | 6 | - | 2 | 27 | - |
| - | - | 8 | - | 3 | 23 | - |
| - | - | - | 2 | 2 | 34 | - |
| - | - | - | 4 | 1 | 33 | - |
| - | - | - | 6 | 1 | 35 | - |
| - | - | - | 8 | - | - | - |
| 2 | - | 2 | - | 1 | 43 | 45 |
| 2 | - | 4 | - | 3 | 32 | 39 |
| 2 | - | 6 | - | 7 | 21 | 32 |
| 2 | - | 8 | - | 4 | 39 | 40 |
| 4 | - | 2 | - | 1 | 44 | 45 |
| 4 | - | 4 | -` | 1 | 45 | 45 |
| 4 | - | 6 | - | 2 | 42 | 54 |
| 4 | - | 8 | - | 1 | 34 | 49 |
| - | 2 | - | 2 | | | |
| - | 2 | - | 4 | 1 | 32 | 112 |
| - | 2 | - | 6 | 1 | 34 | 96 |
| - | 2 | - | 8 | 2 | 28 | - |
| - | 4 | - | 2 | - | - | - |
| - | 4 | - | 4 | - | - | - |
| - | 4 | - | 6 | - | - | - |
| - | 4 | - | 8 | 1 | 26 | 45 |

*Only responding treatments are computed, MS medium containing sucrose (3%) (w/v), NAA: α-naphthalene acetic acid, IAA: Indole 3 acetic acid, BA: Benzyl adenine, KN: Kinetin

Table 7: Effect of different substrata for primary hardening and in the potting mix on survival of regenerates of *P. villosum* var. *boxallii*

| Potting mix composition | Survival (%) (±SE)* | Average plantlet height (cm) |
|---|---|---|
| Coconut husk | 20.1 (2.0) | 6.8 |
| Brick pieces+charcoal chunks+coconut husk (1:1:1) | 40.0 (1.0) | 7.5 |
| Sand+decaying organic matter+brick pieces+charcoal chunks+dried cow dung (1:1:1:1:1) | 65.1 (0.5) | 10.3 |
| Sand+decaying organic matter (1:1) | 58.2 (2.0) | 6.8 |
| Wooden pieces | 10.5 (0.5) | 6.0 |

*Standard error from mean, data represents mean of 3 replicates, data recorded after 20 weeks of transfer

exposed to low intensity sunlight for ~1 h a day for 2 weeks and transplants were fed with 1/10th strength MS liquid solution weekly. Feeding the plantlets with nutrient salt solution has been reported to be beneficial for the promotion of orchid survival and growth[13]. Potting mix containing brick pieces+charcoal chunks+coconut coir (1:1:1) registered 40% survival an average shoot growth of 2.5 cm during the acclimatization process. Burning of leaf tips were observed initially on substrate containing charcoal. Among the different combination of substrata and potting mixes tested, combination of sand, decaying organic matter, brick pieces, charcoal and dried cow dung at 1:1 ratio each was found to be ideal for hardening and subsequent post transplantation survival where 65.1% transplants survived after 2 months of potting with an average shoot growth of 5.3 cm in 5 months (Table 7, Fig. 1k). Well hardened plants were transferred from the orchidarium to the wild.

## DISCUSSION

*In vitro* orchid seed germination is controlled by various factors. In the present study successful sign of seed germination was recorded as swelling of seeds followed by rapture of teast, formation of PLBs. Various factors like developmental age of the seeds/green pods, nutrient medium, quality and quantity of PGRs, organic carbon etc. Effect of developmental age of immature seeds of orchids on successful asymbiotic germination has been reported in many species[14-17]. Different orchid species respond differently on various basal medium and adjuvant and hence homogeneity of a single protocol is not expected across all orchid species. Moreover, *Paphiopedilum* seed germinates relatively slowly due to seed morphological and physiological characteristics which are similar to other terrestrial orchids[18,19]. In the present study too seed pod age was a crucial factor for successful asymbiotic germination and optimum germination was achieved from seeds of ~240 DAP old pods where 60% seeds germinated and formed PLBs. Seeds from <180 DAP pods recorded nodular swellings but very few PLBs were formed and this could be due to the fact that very young orchid ovules do not support good germination as they need time for organogenesis and synthesis of nutrients to occur, moreover the embryos need time to recognize stimulants present in the medium to support germination. Similar opinion was also argued by Zeng *et al.*[19] and Nhut *et al.*[20]. While Long *et al.*[21] opined that the embryo may be too under developed to absorb nutrients from the medium. While mature embryos from >270 DAP pods may have lignified making it slightly hydrophobic and hence longer time for germination. Zhang *et al.*[22] reported that older aged seeds have a greater potential for storage because of a fuller testa and lower water content. Information on embryo development in *Paphiopedilum* species are limited and there are only a few reports on *in vitro* propagation of *Paphiopedilum* viz., *P. insigne*[23], *P. delenatii*[18] and *P. hirsutissimum*, *P. appletonianum, P. armeniacum*[22] etc. Therefore, it is necessary to determine the right developmental age of pod to achieve an optimal germination. The earliest stage at which the embryos can be cultured successfully varies within the orchid genotype and local conditions[5,15,24].

Till the report of Zeng *et al.*[25] 75 studies have been reported on the *in vitro* propagation of *Paphiopedilum* species and cultivars, including about 32 native species and more than 30 hybrids of which seed germination and PLBs formation have been reported in 58 studies. Different species requires different nutrient medium for germination viz., Hypnonex medium for *P. insigne* var. *sandarae*[23], Knudson[10] 'C' medium for *P. delenatii*[20], VW medium for *P. wardii*[19]. In the present study, amongst the 5 basal media tested, optimum germination was achieved on MS medium where 60% seeds responded positively.

In the present study on organic carbon medium only nodular swellings of seeds was observed. For *in vitro* culture of immature embryos, carbohydrates as an energy source in medium have significant effects on seed germination and protocorm development. *Paphiopedilum* seed germination does not occur without sugar[26]. In the present study, three different organic carbon sources (dextrose, glucose and sucrose) were used at varying strengths (1-4%) in MS medium. The requirements of exogenously supplied organic carbon sources vary with nutrient medium, species and developmental stage of the cultured embryos[27]. Sucrose is the most utilized carbon source for seed germination of *Paphiopedilum* orchids[19,28] though Long *et al.*[21] reported a significantly higher germination percentage of *P. villosum* var. *densissimum* on glucose-amended medium. In *P. villosum* var. *boxallii* seed cultured on organic carbon free medium supported only nodular swelling of embryos and

rupturing of the testa without formation of PLBs. Incorporation of at least one organic carbon source was perquisite for successful *in vitro* culture of seeds. Under the given conditions, optimum germination was achieved on MS medium enriched with 3% sucrose where 60% seed registered germination followed by PLBs formation.

Besides organic carbon, role of CW on asymbiotic *in vitro* seed germination and PLBs development of *P. villosum* var. *boxallii* was studied. Organic supplements like CW, banana homogenate, complex additive such as casein hydrolysate, yeast extract etc. are usually used for orchid seed germination. Organic adjuncts have either stimulatory or inhibiting effect on seed germination[19]. The positive effect of CW was reported in *Coleogyne suaveolens*[29], *Taenia latifolia*[17] and argued that PGR like substance (cytokinins) is present in CW. Long *et al.*[21] reported optimum germination of *P. villosum* var. *densissimum* seeds with 10% CW. In the present investigation, use of CW had little or no effect on seed germination compared to CW control medium. However had a promontory effect on early differentiation into PLBs at lower concentrations ($\leq$15%).

Role of AC on orchid seed germination is debatable and there is no consensus for specific ratio of use. Different orchid species respond differently to AC in the medium. Probably first ever attempt to darken culture medium used for orchid seed germination of Native American *Cypripedium* was done[30] in 1943. Ding *et al.*[14] reported promotion of *Paphiopedilum armeniacum* seed germination on AC (2 g $L^{-1}$) enriched medium where 36.1% germination reported against 9.6% on AC control medium. Present study is also in agreement with previous study and AC (0.05-0.1%) supported formation of healthy PLBs but with increase in concentration (0.2-0.5%) it had negative and embryos tend to form nodular swelling only. This effect may be due to absorbing certain compounds including certain vitamins and hormones by AC resulting in a passive effect as also reported by Thomas[31].

In the present study, though initial sign of germination was faster in the dark but germination rate was lower compared to embryos maintained under diffused light (20 µmol $m^{-2}$ $sec^{-1}$). Optimum germination of 60% was recorded under diffused light. Findings of the present study are in agreement with Deb and Temjensangba[15] where diffused light condition was found to be ideal for seed germination of *Malaxis khasiana*.

Different species requires different PGRs for seed germination[32]. Of the 2 PGRs tested at different concentrations in the present study, BA enriched medium found to be better over other PGRs among singly treatments. Synergistic treatment of NAA and BA improved the germination pattern significantly and optimum germination (60%) was achieved on medium fortified with NAA+BA (2.0+6.0 µM in combination) accompanied with PLBs formation with first leaf initials. Synergistic effect of auxin and cytokinin was observed in other orchid species also and the findings of the study is in agreement with Li and Xu[33] where they reported the stimulatory effect of NAA in conjunction with cytokinins in *Rhynchostylis gigantea* using NAA (0.2 mg $L^{-1}$) and BA (0.05 mg $L^{-1}$).

*In vitro* plant regeneration and culture proliferation are affected by different factors viz., quality and quantity of nutrient medium, organic carbon source and quantity, quality and quantity of PGR etc. In the present study MS medium in general found to ideal for plant regeneration and culture proliferation. Chen *et al.*[34], Temjensangba and Deb[13] noted that the change in culture conditions and medium altered the pattern of organogenesis and thus, by altering the nutrient composition desirable response could be achieved. Within 21 days of culture as many as 7 shoot buds developed per explant which subsequently developed leaves with roots and differentiated into rooted plantlets, while, other two media viz., Knudson[10] 'C' and Mitra *et al.*[9] were found to be not ideal for culture proliferation of *Paphiopedilum villosum* var. *boxallii*. Long *et al.*[21] suggested that most *Paphiopedilum* species prefer a low mineral medium for seed germination and the inhibition of *Paphiopedilum* germination on MS medium. However, in the present study low level of nutrient media had an adverse effect on plant regeneration and culture proliferation.

In the present study effect of three organic carbon sources (dextrose, glucose and sucrose, 0-4%) was investigated for culture proliferation. Temjensangba and Deb[13] noted that requirements of the quality and quantity of exogenous supply of the organic carbon sources varies with the species, the media compositions used. Sucrose fortified medium supported optimum differentiation and culture proliferation of *P. villosum* var. *boxallii* while, on dextrose enriched medium recorded stunted root growth though shoot growth was satisfactory (data not presented).

Four different PGRs viz., NAA, IAA, BA and KN were used in the present investigation for plant regeneration culture proliferation and NAA and BA (2 and 6 µM, respectively) in combination found to be beneficial for regeneration and mass multiplication. Similar results were reported by Huang *et al.*[3] for shoot proliferation and rooting in 3 *Paphiopedilum* hybrids viz., *Paphiopedilum philippinense* × *Paphiopedilum* Susan Booth, *Paphiopedilum bellatulum* "Big spot" × *Paphiopedilum* Jo Ann's Wine and *Paphiopedilum* micranthum × *Paphiopedilum glaucophyllum* wherein shoots doubled every 12 weeks when treated with 13 µM BA and 1.6 µM NAA in combination. Nutrient medium fortified with NAA alone did not support PLBs formation while, BA alone supported multiple shoots with elongated leaves

accompanied by poor root growth. At higher concentration of BA (8 µM), explants exhibited extensive necrosis showing an intolerance to the PGR and this finding is in agreement with Long *et al*.[21]. IAA alone supported formation of no/very few PLBs but with time the explants and PLBs degenerated. IAA in combination with KN across studied concentrations had similar inhibitory effect on seedling growth. In the present study optimum results for healthy root and shoot growth without formation of multiple shoots were observed at combination of NAA (4 µM) and BA (8 µM) (Fig. 1j).

Post regeneration *in vitro* hardening of regenerates is necessary for better survival and successful establishment of the regenerates. The survival percentage is hence, finally determined by the hardening of the plantlets. Usually, the tissue culture raised plants are hardened by transferring on a low nutrient medium having low organic carbon sources and maintained at increasing light intensity. About 5 cm long rooted plantlets were hardened by maintaining on different substrata at different combinations. Post transplantation mortality of tissue culture plants is usually attributed to low humidity, high light intensity, transpiration loss, non-sterile condition of the *in vivo* environment etc[35]. The hardened *in vitro* plantlets were then transferred in community pots containing different potting mix combinations. Transplants were exposed to low intensity sunlight for ~1 h a day for 2 weeks and transplants were fed with 1/10th strength MS liquid solution weekly. Feeding the plantlets with nutrient salt solution has been reported to be beneficial for the promotion of orchid survival and growth[13].

## CONCLUSION

*Paphiopedilum* species and their hybrids are the only commercially grown orchids that are not cloned since explants from mature plants are recalcitrant to *in vitro* morphogenesis *Paphiopedilum* available commercially are mostly produced from *in vitro* germinated seeds. *Paphiopedilum villosum* var. *boxallii* is one of the commercially viable species of the genus and there is very little information on propagation of the species is available. Present study presents an efficient protocol for propagation of this vulnerable and economically important species the asymbiotic seed culture and the different factors involved in it. Further, this study also presents the use of some low cost agar alternatives as substrata in the hardening stage which helps in easy establishment of regenerates in the potting mix and field. This protocol may be used by the commercial orchid growers for commercial scale production of this important species.

## SIGNIFICANCE STATEMENT

*Paphiopedilum villosum* var. *boxallii* is a horticultural important vulnerable horticultural important orchid. *In vitro* propagation protocol has been developed from immature embryos and the involvement of different factors. Besides these, low cost agar alternatives were successfully used for *in vitro* hardening which makes the regenerates establish better in the potting mix. Regenerates are successfully established in the nature.

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## REFERENCES

1. Liu, Z.J., S.C. Chen, L.J. Chen and S.P. Lei, 2009. The Genus Paphiopedilum in China. Science Press, Beijing, pp: 13-349.
2. Kumar, P. and H. Rankou, 2015. *Paphiopedilum villosum*. The IUCN Red List of Threatened Species. https://www.iucnredlist.org/
3. Huang, L.C., C.J. Lin, C. Kuo, B.L. Huang and T. Murashige, 2001. *Paphiopedilum* cloning *in vitro*. Scient. Hortic., 91: 111-121.
4. Liao, Y.J., Y.C. Tsai, Y.W. Sun, R.S. Lin and F.S. Wu, 2011. *In vitro* shoot induction and plant regeneration from flower buds in *Paphiopedilum* orchids. *In vitro* Cell. Dev. Biol. Plant, 47: 702-709.
5. Arditti, J., 2008. Micropropagation of Orchids. 2nd Edn., Blackwell Publishing Ltd., Maiden, MA.
6. Chugh, S., S. Guha and I.U. Rao, 2009. Micropropagation of orchids: A review on the potential of different explants. Sci. Hortic., 122: 507-520.
7. Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Planta., 15: 473-497.
8. Gamborg, O.L., R.A. Miller and K. Ojima, 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res., 50: 151-158.
9. Mitra, G.C., R.N. Prasad and A. Roychoudhury, 1976. Inorganic salts & differentiation of protocorms in seed callus of an orchid & correlated changes in its free amino acid content. Ind. J. Exp. Biol., 14: 350-351.
10. Knudson, L., 1946. A new nutrient solution for the germination of orchid seed. Am. Orchid Soc. Bull., 15: 214-217.

11. Schenk, R.U. and A.C. Hildebrandt, 1972. Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can. J. Bot., 50: 199-204.
12. Darlington, C.D. and L.F. La Cour, 1976. The Handling of Chromosomes. 6th Rev. Edn., Georg Allen and Unwin Ltd., London, UK., ISBN-13: 9780045740147, Pages: 201.
13. Temjensangba and C.R. Deb, 2005. Regeneration and mass multiplication of *Arachnis labrosa* (Lindl. ex Paxt.) Reichb: A rare and threatened orchid. Curr. Sci., 88: 1966-1969.
14. Ding, C.C., H. Yu and F.Y. Liu, 2004. Factors affecting the germination of *Paphiopedilum armeniacum*. Acta Botanica Yunnanica, 26: 673-677.
15. Deb, C.R. and Temjensangba, 2006. *In vitro* propagation of threatened terrestrial orchid, *Malaxis Khasiana* Soland ex. Swartz through immature seed culture. Indian J. Exp. Biol., 44: 762-766.
16. Deb, C.R. and Temjensangba, 2007. Rapid mass multiplication of *Cleisostoma racemiferum* (Lindl.) Garay: An endangered orchid. J. Plant Biol., 34: 99-105.
17. Deb, C.R. and Sungkumlong, 2008. *In vitro* regeneration and mass multiplication of *Taenia latifolia* (Lindl.) using immature seeds a threatened terrestrial orchid. J. Plant Biol., 35: 1-6.
18. Lee, Y.I., E.C. Yeung, N. Lee and M.C. Chung, 2006. Embryo development in the lady's slipper orchid, *Paphiopedilum delenatii*, with emphasis on the ultrastructure of the suspensor. Ann. Bot., 98: 1311-1319.
19. Zeng, S., K. Wu, J.A.T. da Silva, J. Zhang, Z. Chen, N. Xia and J. Duan, 2012. Asymbiotic seed germination, seedling development and reintroduction of *Paphiopedilum wardii* Sumerh., an endangered terrestrial orchid. Scient. Hortic., 138: 198-209.
20. Nhut, D.T., P.T.T. Trang, N.H. Vu, D.T.T. Thuy, D. Van Khiem, N. Van Binh and K.T.T. Van, 2005. A wounding method and liquid culture in *Paphiopedilum delenatii* propagation. Propag. Ornam. Plants, 5: 158-163.
21. Long, B., A.X. Niemiera, Z.Y. Cheng, C.L. Long, 2010. *In vitro* propagation of four threatened *Paphiopedilum* species (Orchidaceae). Plant Cell Tissue Organ Cult., 101: 151-162.
22. Zhang, J.J., N. Yan and H. Hu, 2013. The seed development of three *Paphiopedilum* species in relation to asymbiotic germination. Plant Diversity Resourc., 35: 33-40.
23. Nagashima, T., 1982. Studies in the seed germination and embryogenesis in *Goeringii* Rchb. F. and *Paphiopedilum insigne* var. sanderae Rchb. J. Jpn. Soc. Hort. Sci., 51: 94-105.
24. Sauleda, R.P., 1976. Harvesting times of orchid seed capsules for the green pod culture process. Am. Orchid Soc. Bull., 45: 305-308.
25. Zeng, S.J., W. Huang, K. Wu, J. Zhang, J.A.T. da Silva and J. Duan, 2016. *In vitro* propagation of *Paphiopedilum* orchids. Crit. Rev. Biotechnol., 36: 521-534.
26. Pierik, R.L.M., P.A. Sprenkels, B. van der Harst and Q.G. Van der Meys, 1988. Seed germination and further development of plantlets of *Paphiopedilum ciliolare* Pfitz. *in vitro*. Scient. Hortic., 34: 139-153.
27. Temjensangba and C.R. Deb, 2006. Effect of different factors on non-symbiotic seed germination, formation of protocorm-like bodies and plantlet morphology of *Cleisostoma racemiferum* (Lindl.) Garay. Indian J. Biotechnol., 5: 223-228.
28. Hossain, M.M., R. Kant, P.T. Van, B. Winarto, S.J. Zeng and J.A.T. da Silva, 2013. The application of biotechnology to orchids. Crit. Rev. Plant Sci., 32: 69-139.
29. Sungkumlong and C.R. Deb, 2008. Effects of different factors on immature embryo culture, PLBs differentiation and rapid mass multiplication of *Coelogyne suaveolens* (Lindl.) Hook. Indian J. Exp. Biol., 46: 243-248.
30. Curtis, J.T., 1943. Germination and seedling development in five species of *Cypripedium* L. Am. J. Bot., 30: 199-206.
31. Thomas, T.D., 2008. The role of activated charcoal in plant tissue culture. Biotechnol. Adv., 26: 618-631.
32. Da Silva, J.A.T., 2013. Orchids: Advances in tissue culture, genetics, phytochemistry and transgenic biotechnology. Floriculture Ornamental Biotechnol., 7: 1-52.
33. Li, Z.Y. and L. Xu, 2009. *In vitro* propagation of white-flower mutant of *Rhynchostylis gigantea* (Lindl.) Ridl. through immature seed-derived protocorm-like bodies. J. Hortic. For., 1: 093-097.
34. Chen, Z.L., X.L. Ye, C.Y. Liang and J. Duan, 2004. Seed germination *in vitro* of *Paphiopedilum armeniacum* and *P. micranthum*. Acta Hortic. Sin., 31: 540-542.
35. Deb, C.R. and T. Imchen, 2010. An efficient *in vitro* hardening technique of tissue culture raised plants. Biotechnology, 9: 79-83.