Journal of Bioresources 9 (2): 26–32, July – December 2022

JOURNAL OF BIORESOURCES

ISSN: 2394-4315 (Print)
ISSN: 2582-2276 (Online)

journal webpage: http://jbr.rgu.ac.in

RESEARCH ARTICLE

Asymbiotic *in vitro* seed culture of *Paphiopedilum fairrieanum*, *P. venustum* and *Dendrobium densiflorum* on low-cost substrata

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Article No.: RKSJBR51; Received 26.10.2022; Reviewed: 24.11.2022; Accepted: 15.12.2022; Published: 31.12.2022
Doi: https://doi.org/10.5281/zenodo.8251896

Abstract

Asymbiotic germination of immature seeds of orchids represents a valuable method for large-scale seedling production, addressing both commercial demand and the conservation of rare and endangered species. In this study, we explored the efficacy of three low-cost substrates: Polyurethane foam (PUF), Coconut husk chips (CHC), and Tree Leaf litter (TLL), as substitutes for the expensive agar-gelled medium in the *in vitro* seedling culture of three commercially important orchid species, namely, *Paphiopedilum fairrieanum* (Lindl.) Stein., *P. venustum* (Wall. ex Sims) Pfitzer, both endangered Slipper orchids, and *Dendrobium densiflorum* Lindl. ex Wall. During a 120-day culture period, we recorded germination time, germination frequency, and the proportion of different growth stages on these substrates, comparing them to the agar-gelled medium. All substrates were fortified with half Murashige and Skoog medium, 0.5 mg/L α-Napthaleneacetic acid, 10% Coconut water, and 2% Sucrose. Subsequently, *in vitro* hardening of rooted seedlings was conducted using composites prepared from cost-effective materials, including charcoal, brick pieces, and coconut husk chips. Further, secondary hardening was done on the same composite that additionally contained garden soil, sand, and a top cover of moss. Among the tested substrates, agar-gelled medium exhibited the highest seed germination and seedling formation for *Paphiopedilum*, while it performed equally well as PUF for *D. densiflorum*. Conversely, the germination time for all species was one week shorter when using PUF. In comparison, CHC resulted in a lower germination frequency and prolonged germination time. Although protocorms (stage 3) of all species developed maximally on CHC, only *D. densiflorum* reached the rooted seedling stage. The performance of TLL was significantly inferior for all species, with none of them progressing to protocorm (stage 3). After primary hardening, ~80% of seedlings of *P. fairrieanum* and *D. densiflorum*, and ~70% of seedlings of *P. venustum* survived. Following 90 days of secondary hardening inside a 70% shade net house, about two-thirds, three-fourths, and half of the total transplants of *P. fairrieanum*, *P. venustum*, and *D. densiflorum*, respectively, thrived in the pots. Our findings suggest that PUF presents a cost-effective alternative for cultivating *P. fairrieanum*, *P. venustum*, and *D. densiflorum*. Additionally, CHC holds promise for low-cost production of their protocorms, which, upon multiplication on suitable media, could be developed into a large number of seedlings.

Keywords: Orchids; Endangered; Commercial; *In vitro* Multiplication; Micropropagation; Non-gelling Substrata; Protocorm; Seedling Development

1. Introduction

Orchids stand as one of the most prized floriculture products due to their exquisite floral beauty and prolonged shelf life, traded both as cut flowers and potted plants in national and international markets. About 1300 orchid species belonging to 190 genera have been reported from India and that includes 972 species (168 genera) from Northeast India and 577 species (148 genera) alone from Arunachal Pradesh (Rao, 2018). Unfortunately, rampant habitat destruction, land-use changes, unregulated harvesting, and climate change have contributed to the decline of many orchid species in the wild (Fay, 2018). Consequently, a significant number of orchid species are categorized as critically endangered, endangered, vulnerable, and threatened, leading to restrictions on their international trade from wild sources as per the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 2021). Orchids produce numerous dust-like, tiny seeds that have a low rate of *in vivo* germination due to their dependence on mycorrhizal association and specific vegetational niches for successful germination (Dearnaley, 2007; Favre-Godal et al., 2020; Kolanowska et al., 2020). Consequently, habitat-specialist orchids with narrow ecological amplitudes, compared to generalist species, exhibit slow recovery and self-replenishment in pre-disturbed forests (Besi et al., 2019; Martín-Forés et al., 2022; Evans and Jacquemyn, 2022).

Paphiopedilum Pfitzer, widely known as the 'Slipper orchid,' represents one such terrestrial genus critically endangered according to IUCN (2017) and CITES (2021), receiving legal protection in India (Deb and Jakha, 2019). Native to tropical Asia, this genus comprises approximately 96-100 species, including nine species recorded in India. *Paphiopedilum* hold immense value in the ornamental market due to their uniquely structured, multi-colored, long-lasting flowers (60-90 days) (Rao, 2006), along with attractive evergreen foliage produced by certain species (Cribb, 1998; Hong et al., 2008). However, large-scale production of *Paphiopedilum* is primarily achieved through *in vitro* asymbiotic germination of immature seeds, as mature plant-derived explants are recalcitrant to shoot induction and plant regeneration, often suffering from severe endogenous bacterial contamination (Deb and Jakha, 2020).

In vitro asymbiotic seed germination on agar-based nutrient medium is a commonly employed technique for large-scale production of orchid seedlings to meet market demands and support conservation efforts (Pongener and Deb, 2019). Nevertheless, agar, the most widely used solidifying agent in plant tissue culture media, has its limitations, including high cost and other undesirable characteristics (Babbar and Jain, 1998; Bhattacharya et al., 1994; Ebile et al., 2022). To optimize seedling production and reduce costs, researchers have explored efficacy of various gelling and non-gelling agents as low-cost agar alternatives for multiple plant species, including orchids, yielding varying levels of success (Ebile et al., 2022; Deb and Pongener, 2022). Among non-gelling agents, Polyurethane foam (PUF) and Coconut coir/husk have been recognized as suitable substrates for certain orchid species. PUF, owing to its lightweight, spongy, and resilient structure, effectively absorbs an aqueous medium, meeting fundamental requirements in *in vitro* plant tissue culture systems, and additionally serving as a beneficial raft material for root

immobilization (Kawka et al., 2021). Its low surface-area nature exhibits remarkably high sorption capacities (Han et al., 2015).
In this study, we aimed to economize the production cost of orchids by investigating the potential of low-cost non-gelling substrates for *in vitro* asymbiotic seed germination, seedling formation, as well as subsequent hardening and acclimatization. We focused on three commercially important orchids from Arunachal Pradesh: *Paphiopedilum fairrieanum* (Lindl.) Stein., a critically endangered and endemic orchid of Eastern Himalaya (Rankou and Kumar, 2015), *P. venustum* (Wall. ex Sims) Pfitzer, an endemic and endangered orchid of Northeast India (Rankou and Kumar, 2015; Kaur and Bhutani, 2016), and *Dendrobium densiflorum* Lindl. ex Wall. (a medicinal epiphyte and splendid ornamental species among the *Dendrobium* genus). For *in vitro* seed germination and seedling development, we employed three low-cost materials, namely Polyurethane foam, Coconut husk chips, and Tree Leaf litter as non-gelling substrates, while a composite comprising charcoal, brick pieces, coconut husk chips, and garden soil was examined for *in vitro* and *ex vitro* hardening and acclimatization of the plantlets. This paper highlights the merits and demerits of these low-cost substrates in orchid cultivation.

2. Materials and methods

2.1. Capsule source and sterilization

Naturally pollinated immature green capsules of *Paphiopedilum fairrieanum*, *P. venustum* and *D. densiflorum* were collected from orchid orchard maintained by State Forest Research Forest (SFRI) at Dirang and Itanagar (Arunachal Pradesh). The approximate age of the capsules of the above species at the time of collection was about 240 – 270, 210 – 240 and 150 – 180 days after pollination (DAP), respectively. The capsules were carefully scrubbed with a laboratory detergent called “Extran” (1:100 v/v; MA 02, Merck) to remove dust and then thoroughly rinsed under clean running water to eliminate traces of the detergent. Subsequently, the capsules were surface-sterilized with an aqueous solution of 0.05% Tween 20 and 0.1% Mercuric chloride for 10 min. under a laminar air cabinet, followed by washing with sterilized double-distilled water until all foam was removed. The capsules were then sprayed with 70% ethanol, quickly flamed off, and longitudinally cut with a sterile blade to scoop out seeds in sterile petridishes for raising culture.

2.2. Preparation of substrata for orchid culture

Polyurethane Foam (PUF) obtained from the local market, Coconut Husk Chips (CHC) prepared from dry ripened fruits, and Fresh Tree Leaf Litter (TLL) collected from the Botanical Garden were used as agar alternatives. PUF was appropriately cut to fit into culture tubes, while CHC and TLL were chopped into small pieces (5 mm size), soaked in a 1:100 v/v solution of "Extran" for 6 hrs, and thoroughly rinsed with clean water. All these substrata were air-dried, autoclaved at 15 psi for 15 min., and then stored for further use (Pongener and Deb, 2019).

2.3. Culture media and conditions for asymbiotic seed germination

In vitro asymbiotic seed germination was conducted using agar-gelled half Murashige and Skoog medium (½ MS) supplemented with 0.5 mg/L α-Napthaleneacetic acid (NAA) and 10% Coconut water (v/v, CW). Sucrose (2% w/v) and Agar (0.8% w/v) were added to the medium (Zeng et al., 2012). A volume of 15 ml of liquid medium was dispensed into each culture tube (size: 25 mm x 150 mm). For other substrata, the same quantity, except agar, was dispensed into the culture tubes (Deb and Pongener, 2013). The pH of the media was adjusted to 5.8±0.5 with 1N NaOH/1N HCl before autoclaving at 15 psi for 20 min.

The seeds were scooped out with a spatula (0.5 mm wide) and inoculated onto agar-gelled medium and other substrata. Culture tubes of *Paphiopedilum* were incubated in the dark for 45 days, whereas those of *D. densiflorum* were incubated for 15 days. Afterward, the cultures were provided with a 16/8 h photoperiod under cool white light at 40 µmol $m^{-2}s^{-1}$ and maintained at 24±2 °C.

2.4. Sub-culturing and in vitro- and ex vitro hardening of plantlets

Eighteen healthy seedlings (20-30 mm length) were taken out from each substratum after 120 days and sub-cultured (3 seedlings per tube) on ½MS agar-gelled (0.8% w/v) growth medium supplemented with NAA (0.5 mg/L), CW (10%) and sucrose 2% (w/v). After 60 days of sub-culturing, four seedlings, each measuring 35 to 45 mm in length, were transferred into six separate culture containers for a 30-day period of in vitro primary hardening. This hardening process occurred on a matrix consisting of a combination of charcoal pieces, brick pieces, and CHC (1:1:1). This matrix was kept suitably moistened using a solution of ⅒ MS, as described by Pongener and Deb (2019). Surviving seedlings were then transferred to a secondary hardening stage by transplanting them into plastic cups filled with a potting mixture of garden soil, sand, charcoal, and brick (2:1:1:1 v/v) and covered with Sphagnum moss. These seedlings were covered with perforated plastic bags and kept under a 70% shade net for 30 days. Finally, well-established plantlets were grown in a natural environment for the next 60 days and monitored for survivability.

2.5. Experimental design and data analysis

The experimental design was completely randomized, and all the treatments consisted of six culture tubes as replicates. Experiments were repeated twice. Cultures were visually observed after 7 days of inoculation to record the seed germination time. After 120 days of inoculation, the germinated seeds from three 1.0 cm^2 grids were randomly scooped out from each tube and dispersed over a glass slide (Roy et al., 2011). Various developmental stages of seedlings were observed and counted under a stereomicroscope (Stemi 508, Carl Zeiss), and their proportions were calculated by dividing the number of seeds in a given stage by the total number of seeds multiplied by 100.

Seed germination and seedling development growth stages of orchid (Zeng et al., 2012)

Stage	*Description*
0	Ungerminated seed with embryo and no rupturing of the testa
1	Rupture of the testa by enlarging embryo (= germination)
2	Appearance of the shoot (= promeristem) and /rhizoids
3	Emergence and elongation of the first leaf
4	One leaf and one or more roots present
5	Presence of two or more leaves, roots present (= seedling)

Data were analyzed using one-way ANOVA with SPSS 21 version, and standard error of the mean was calculated. Means were further compared using Duncan Multiple Range Test at p = 0.05.

3. Results

In order to find out alternative substrata suitable for low-cost orchid production, immature seeds of *P. fairrieanum, P. venustum* and *D. densiflorum* were asymbiotically germinated on three different non-gelling substrata (PUF, CHC and TLL) and agar-gelled medium as control. All substrata were supplemented with ½ MS medium containing NAA (0.5 mg/L), CW (10%), and Sucrose (2%). The progression of seeds (stage 0) to different developmental stages (1-5) was recorded after 120 days of culture (Figure 1). The

Table 1: Survival rate of seedlings of different orchid species after sub-culturing for 60 days on fortified ½ MS agar-gelled growth medium

Orchid species	Survival rate (%) of Seedling derived from different substrata		
	Agar	PUF	CHC
P. fairrieanum	94.4±5.6	94.4±5.6	-
P. venustum	88.9±7.0	88.9±7.0	-
D. densiflorum	94.4±5.6	94.4±5.6	88.9±7.0

Values are Mean ± SEM. Effect of substrata was not significant (p>0.05) for any species.

Table 2: Survival rate of seedlings of different orchid species after Primary and Secondary hardening

Orchid species	Survival rate (%) of seedlings	
	In vitro hardening (30 days)	*Ex vitro* hardening (90 days)
P. fairrieanum	83.3±5.3	70.0±10.9
P. venustum	70.8±7.7	70.6±12.0
D. densiflorum	83.3±10.5	50.0±12.9

Values are Mean ± SEM.

seed germination time of all the orchid species on three substrata except TLL, ranged between 23-79 days. TLL proved to be an inferior substratum, with seeds germinating in 70-92 days (Figure 2a). Among the substrata, germination was relatively early on PUF followed by agar and CHC ($p<0.05$). On each substratum, seed

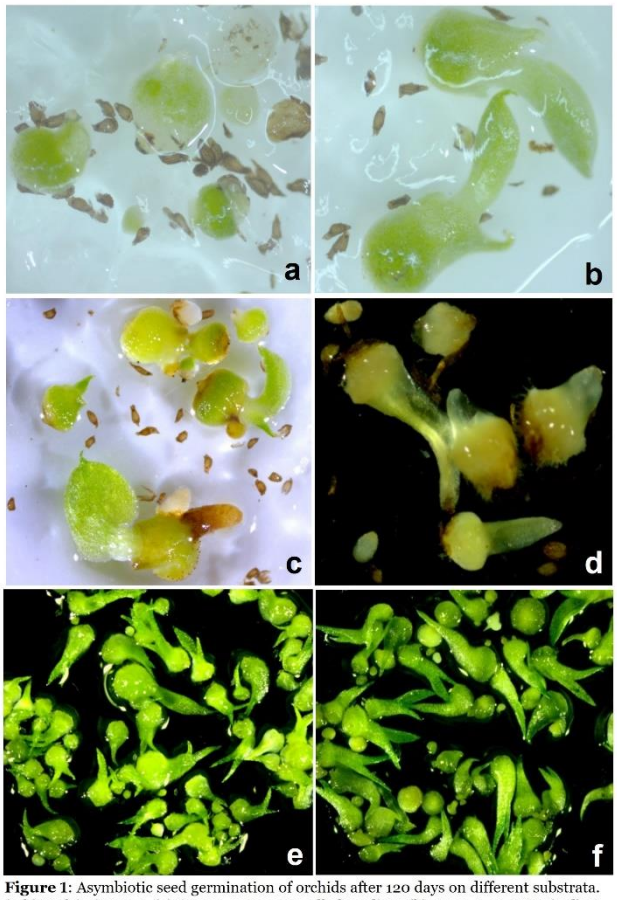

Figure 1: Asymbiotic seed germination of orchids after 120 days on different substrata. (a-b) *P. fairrieanum* (a) Stage 1-3 on agar-gelled medium (b) Stage 4 on PUF; (c-d) *P. venustum* (c) Stage 1-4 on agar-gelled medium (d) Stage 1-4 on PUF; (e-f) *D. densiflorum* (e) Stage 4-5 on agar-gelled medium, (f) Stage 4-5 on PUF

germination of *D. densiflorum* occurred earlier than *P. fairrieanum* and *P. venustum*, whose germination time was virtually the same.

Swelling (Stage 1) followed by testa burst (stage 2) occurred in inoculated seeds of all three orchid species on each substratum. Seed germination rate (sum total proportion of Stage 0 to 5) was maximum on agar and minimum on TLL (Figure 2h). However, there was a marked difference between the two orchid genera. Seeds of *D. densiflorum* germinated relatively more on every substratum than *Paphiopedilum* spp. The germination rate of *D. densiflorum*, *P. fairrieanum* and *P. venustum* on agar was 84.7%, 78.7% and 74.3% respectively, followed by PUF (82.4%, 67.9% and 65.1%), CHC (78.5%, 50.3% and 43.2%), and TLL (38.3%, 19.3% and 17.6%), showing a significant difference among the media towards growth promotion. On the later substratum, the germinated seeds of *Paphiopedilum* spp. and *D. densiflorum* could only reach up to stage 1 and stage 2, respectively, and did not develop into protocorm even after 120 days of culture (Figure 2c-e).

CHC did not appear to be a viable substratum for *Paphiopedilum* species, as both of them could not progress beyond stage 3. However, it proved to be a viable option for *D. densiflorum* which could progress to stage 4 (15.9%) and stage 5 (10.7%) after 120 days of culture (Figure 2f-g).

Seedlings (stage 4 and 5) of all three orchid species developed on both agar and PUF- supported media. However, there was a significant difference between the two media for *Paphiopedilum* spp. On agar, both *P. fairrieanum* and *P. venustum* formed proportionately more seedling of stage 4 (9.3% and 7.1%, respectively) and stage 5 (8.2% and 4.8%, respectively), whereas on PUF, the numbers were only 3.9% and 3.6% for stage 4 and 3.5% and 3.6% for stage 5, respectively (Figure 2f-g). However, in case of *D. densiflorum*, the rate of morphogenesis to stage 5 was statistically equal on both media (13.7% and 12.0%), but stage 4 was significantly more on PUF (25.7% vs 29.0%). It was also found that, in case of *D. densiflorum*, the rate of seedling formation (both stage 4 and 5) on agar, PUF and CHC supported media was much higher in comparison to *Paphiopedilum* spp. (Figure 2f-g).

Stage 5 seedlings of all orchid species that developed on each substratum were sub-cultured on the same ½ MS medium containing NAA (0.5 mg/L) and CW (10 %) for further growth. After 60 days of sub-culturing, 89-94% seedlings of *P. fairrieanum*, *P. venustum* and *D. densiflorum* obtained from different substrata survived (Table 1, Figure 3). Afterwards, twenty-four seedlings of each species pooled together from different substrata were transferred for primary hardening on a matrix composed of Charcoal pieces, Brick pieces and CHC (1:1:1, v/v). After 30 days of transfer, 83.3% seedlings each of *P. fairrieanum* and *D. densiflorum*, and 70.8% seedlings of *P. venustum* survived (Table 2, Figure 3). Subsequently, all surviving seedlings of each species were transplanted for secondary hardening on a potting mixture prepared from Garden soil, Charcoal pieces, Brick pieces and Sand (2:1:1:1, v/v). After 90 days of transfer, 70.0%, 70.6% and 50% seedlings, respectively, of *P. fairrieanum*, *P. venustum* and *D. densiflorum* thrived in the pots (Table 2).

4. Discussion

The objective of this study was to explore low-cost substrata as potential alternatives to agar in orchid tissue culture and primary hardening of seedlings for three commercially important orchids: *Paphiopedilum fairrieanum*, *P. venustum*, and *Dendrobium densiflorum*. The survival of these seedlings during secondary hardening was also assessed. Asymbiotic seed germination and seedling development were conducted using PUF, CHC, and TLL as low-cost non-gelling substrata, along with agar-gelled medium, all fortified with ½ MS solution containing 0.5 mg/L NAA, 10% CW and 2% sucrose.

The results showed that germination frequency, initiation time, and proportions of protocorm and seedling formation during the 120-day period varied with different substrata as well as the orchid genera. Germination experiments conducted on 240–270 DAP seeds of *P. fairrieanum*, 210–240 DAP seeds of *P. venustum* and 150–180 DAP seeds of *D. densiflorum* showed the highest germination frequency of ~75% in *Paphiopedilum* and ~85% in *D. densiflorum* on fortified ½ MS agar-gelled medium (Figure 2f-g). Asymbiotic germination of fully mature orchid seeds is considered often difficult due to formation of a lignified testa, and the germination frequency is reported to drop beyond certain level of seed maturity (Pieriket al., 1988; Lee et al., 2006; Zeng et al., 2016). Mao and Ranyaphi (2013) reported that in six species of *Paphiopedilum* (*P. fairrieanum*, *P. hirsutissimum*, *P. insigne*, *P. spicerianum*, *P. venustum* and *P. villosum*), the time taken for *in vitro* seed germination varied from 4-9 weeks depending upon the species, and the seeds from immature 6 months old capsules germinated faster than the mature 10 months old capsules. Kaur and Bhutani (2016) made similar observations in case of *P. venustum* that 83% seed from undehisced capsules germinated readily on modified terrestrial orchid medium whereas, only 20% seeds from dehisced, dry, brown capsules could germinate and that too in a comparatively longer time. Besides lignified testa, high concentration of abscisic acid present in very young seeds has also been reported to inhibit asymbiotic germination in case of *P. armeniacum* (Xu et al., 2020). Therefore, high germination frequency recorded in the present study indicates that during this period (DAP) the embryos of these orchid species were well developed, however, their testa was yet not lignified, and the seeds were amply permeable to water and nutrients (Long et al., 2010; Zeng et al., 2014). It is also known that the influence of seed maturity on germination frequency is species-specific (Long et al., 2010) and the optimum seed age for high germination varies from 90 – (120-180) - 400 DAP in different species of *Paphiopedilum* (Lee 2007; Long et al., 2010; Chen et al., 2015; Zeng et al., 2016; Yao et al., 2021). For various *Dendrobium* species, the optimum seed age for maximum germination is reported to range from (120-180) – 240 – 270 DAP (Paul et al., 2012; Teixeira da Silva et al., 2015; Longchar and Deb, 2022). It has also been observed in many cases that germination frequency of orchids is influenced by growth

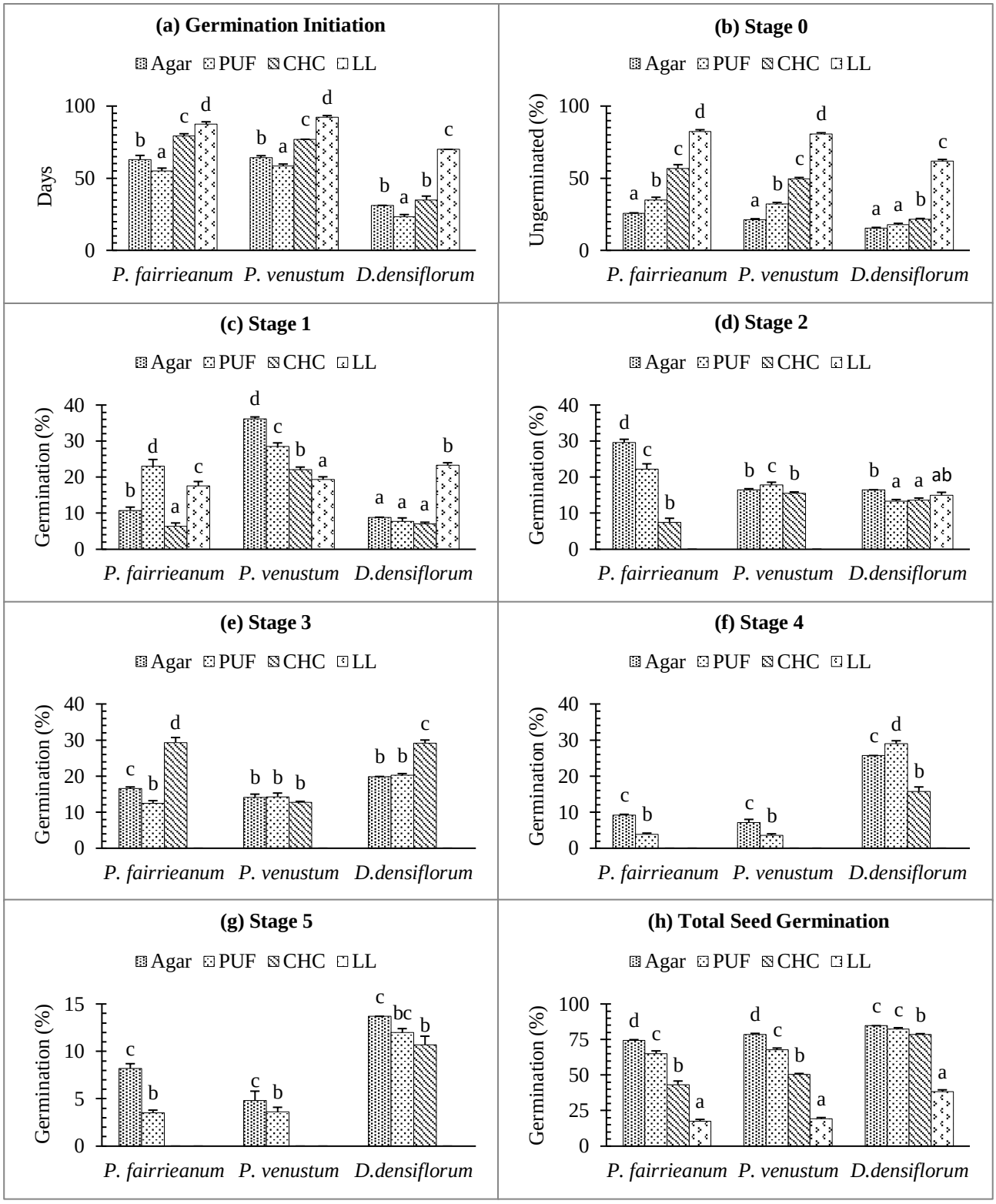

Figure 2: Effect of different substrata supplemented with ½ MS, 0.5 mgL^{-1} NAA, 10% CW and 2% Sucrose on seed germination and development for 120-day culture of three orchid species. Values (Mean ± SEM) followed by different letters on top of columns for each orchid species are significantly different at $p<0.05$ (DMRT).

media (low salts concentration and optimum level of organic additives and growth regulators) and optimum germination conditions (Arditti, 1967; Kauth et al., 2008; Zeng et al., 2014; Teixeira da Silva et al., 2015; Zeng et al., 2016). Seed treatment with NaOCl or NaOH also enhances seed germination (Lee 2007; Zeng et al., 2012; Chen et al., 2015).

On PUF, ~65% of seeds from both *Paphiopedilum* species and ~78% of seeds from *D. densiflorum* germinated, and a good number of them formed protocorms and developed into seedlings (Figure 2e-g). Thus, PUF proved to be as effective as agar on these counts for *D. densiflorum*. However, its effect for *Paphiopedilums*, though satisfactory, was significantly lesser than the agar. On the other hand, seed germination in *Paphiopedilum* and *Dendrobium* began on PUF about one week earlier than on agar, with germination initiation times of 8 and 3 weeks, respectively.

CHC appeared to be good enough substratum for seedling production for *D. densiflorum* but not for the Paphiopedilums, as their germinated seeds could develop maximally up to protocorm (stage 3), despite a fair enough germination frequency (Figure 2e, h).

The germination initiation time of seeds on this substratum was almost the same as on agar-gelled medium for *D. densiflorum* but about 3 weeks delayed for *Paphiopedilum* (Figure 2a).

As a substratum, PUF has been adjudged as good as agar-gelled medium for tissue culturing of *D. densiflorum*, and development of well-rooted healthy plantlets with their velamenous roots attached to its surface has also been observed for some other orchids (Deb and Pongener, 2010, 2013, 2022). Slightly superior performance of PUF in comparison to both agar and coconut coir for *Cymbidium aloifolium* but slightly inferior performance to agar yet superior to coconut coir for *Cymbidium iridioides* has been reported by Deb and Pongener (2010, 2013). We observed that among the three orchids, seed germination of *D. densiflorum* was always more irrespective of substrata and occurred in just 3-5 weeks. High seed germination frequency *in vitro* has been reported in several *Dendrobium* species (Teixeira da Silva et al., 2015). Quicker seed germination in *D. densiflorum* occurring in 3 weeks on both agar-gelled and PUF substrata, and two week early germination on PUF and coconut coir in comparison to agar-gelled medium by two species of *Cymbidium* have been reported (Deb and Pongener, 2010, 2013, 2022). Aggarwal and Nirmala (2012) have also highlighted the benefits of coir matrix which supported early greening of the germinated seeds and higher rate of protocorm multiplication in *Cymbidium pendulum*.

We found seed germination frequency of all the orchid species on TLL to be very low in proportion, taking very long period in germination and not progressing to higher stage of morphogenesis. Germination frequency, germination initiation time and the highest growth stage attained by *Paphiopedilum* and *D. densiflorum* on this substratum was ~1/5th and ~2/5th, 13 weeks and 10 weeks, and stage 1 (rupturing of testa) and stage 2 (promeristem) respectively (Figure 1). Deb and Pongener (2010, 2013) also reported leaf litter as inferior substratum on which seedling of *Cymbidium* did not develop despite 50% seed germination occurring within 7 weeks.

Based on the results obtained, PUF stands out to be a low-cost agar alternative for orchid tissue culture. CHC may be useful for production of protocorms (stage 3) of all these orchid species as a fairly good percentage of germinated seeds developed into Protocorm that could be used as explants for their multiplication by sub-culturing on suitable media and further morphogenesis into seedlings. These substrata have additional advantages over agar as they provide physical support to the protocorms and seedlings by providing attachment sites for the rhizoids and roots, have water absorption and retention properties, allow nutrient replenishment (Deb and Pongener, 2022), and proper aeration necessary for gaseous exchange, and could also be reused for the same purpose (Kawka et al., 2021; Deb and Pongener, 2022).

In vitro raised orchid seedlings require stepwise and careful procedure while being transferred to *ex vitro* condition (Diengdoh et al., 2017) and several protocols have been used for their efficient acclimatization/primary hardening (Deb et al., 2022) and secondary hardening (Deb and Pongener, 2022). Keeping this aspect in view, we first sub-cultured the rooted seedlings produced on different substrata on ½ MS agar medium fortified with same concentration of NAA, CW and sucrose for further development of shoots and roots so that upon transfer for primary hardening the seedlings could withstand the harsh conditions imposed by non-agar matrix and get properly established on composite matrix. As a result, ~80% seedlings of *P. fairrieanum* and *D. densiflorum* each, and ~70% seedlings of *P. venustum* survived after 30 days of primary hardening (Table 2). After secondary hardening, two-third, three-fourth and half of the total transplants of *P. fairrieanum, P. venustum and D. densiflorum* respectively thrived in pots after 90 days (Table 2). Composites prepared from Charcoal, Brick pieces and decayed organic matters have been reported to be effective for proper acclimatization and hardening of orchid seedlings. Deb and Jakha (2019) used nutrient regime similar to our study for *P. villosum* var. boxallii on a composite matrix consisted of charcoal, brick pieces, sand, decaying organic matters, dried cow dung in equal ratio and recorded ~65% survival of transplants in the greenhouse condition. Likewise, composite of charcoal and brick pieces for proper acclimatization and hardening of epiphytic orchid *Cymbidium pendulum* (Aggarwal and Nirmala, 2012), and the same with vermicompost for *C. mastersii* (Mohanty et al., 2012); coconut husk and wood bark for *Dendrobium heterocarpum* (Longchar and Deb, 2022); charcoal and brick pieces topped with moss for *D. hookerianum* (Paul et al., 2012) and D. *crepidatum* (Bhattacharyya et al., 2016); cocopeat, pine bark and sphagnum moss for *D. densiflorum* (Pant et al., 2022); brick pieces, charcoal pieces, coco peat and vermiculite for *D. aphyllum* (Hossain et al., 2013); and charcoal, brick pieces and decayed wood and moss mix for primary hardening (Deb et al., 2022) have been recommended as suitable substrata.

Figure 3: (a-c) *P. fairrieanum* (a) Seedling subculture (b) Primary hardening (c) Secondary hardening; (d-f) *P. venustum* (d) Seedling subculture (e) Primary hardening (f) Secondary hardening; (g-i) *D. densiflorum* (g) Seedling subculture (h) Primary hardening (i) Secondary hardening

5. Conclusion

The aim of the study was to explore the suitability of non-agar low-cost substrata for asymbiotic germination and *in vitro* seedling production of *Paphiopedilum fairrieanum*, *P. venustum* and *Dendrobium densiflorum* as well as to test the effectiveness of a composite prepared from other low-cost substrata for acclimatization and primary hardening of rooted seedlings, followed by secondary hardening of transplanted seedlings. The results indicate that Polyurethane foam is a viable alternative for *Paphiopedilum* species, and Coconut husk chips show promise for *D. densiflorum*. Charcoal, brick and Coconut husk chips based low-cost substrata proves to be a worthy option for the hardening of *Paphiopedilums* and *D. densiflorum*, and might also be used for other orchids as well. The utilization of such such cost-effective materials would not only contribute to large-scale seedling production for conservation purposes but also meet the rising demand for commercially important orchids.

Acknowledgements

The authors are thankful to the Rajiv Gandhi University for providing necessary infrastructure to conduct this investigation. Facilities received from UGC-CPE, DST-FIST, DBT-Institutional Biotech Hub, DBT-DeLCON and UGC-CPE at the University, and Rajiv Gandhi National Fellowship to the first author from Ministry of Tribal Affairs are duly acknowledged.
Funding Source: This work was financially supported by the Department of Biotechnology, Government of India, under grants DBT/NER/Agri/23/2013 and BT/32/NE/2013, both sanctioned in the year 2016.

Authors' contributions

Bengia Mamu: Conceived, designed and performed the research, wrote the draft manuscript; Oyi Dai Nimasow: Supervised the work; Rajiv Kumar Singh: Mobilized funds, supervised the work, conducted statistical analyses, and reviewed the manuscript.
All authors have thoroughly read and agreed to the final version of the manuscript.

Conflict of interests

The authors declare that there is no conflict of interest.

References

Aggarwal Sand Nirmala C. 2012. Utilization of coir fibers as an eco-friendly substitute for costly gelling agents for *in vitro* orchid seed germination. Scientia Horticulturae 133: 89-92. doi:10.1016/j.scienta.2011.10.010

Arditti J. 1967. Factors affecting the germination of orchid seeds. Botanical Review 33: 1-97.

Babbar SB and Jain N. 1998. 'Isubgol' as an alternative gelling agent for plant tissue culture media. Plant Cell Reports17: 318-322.

Besi EE, Nikong D, Mustafa M and Go R. 2019. Orchid diversity in antropogenic-induced degraded tropical rainforest, an extrapolation towards conservation. Lankesteriana: International Journal on Orchidology 19: 107-124. doi.org/10.15517/lank.v19i2.38775

Bhattacharya P, Dey S and Bhattacharyya BC. 1994. Use of low-cost gelling agents and support matrices for industrial scale plant tissue culture. Plant Cell, Tissue and Organ Culture 37: 15-23.

Bhattacharyya P, Kumaria S, Joba N and Tandon P. 2016. *En-masse* production of elite clones of *Dendrobium crepidatum*: A threatened, medicinal orchid used in Traditional Chinese Medicine (TCM). Journal of Applied Research on Medicinal and Aromatic Plants 3: 168-76. doi.org/10.1016/j.jarmap.2016.04.001

Chen Y, Goodale UM, Fan XL and Gao JY. 2015. Asymbiotic seed germination and *in vitro* seedling development of *Paphiopedilum spicerianum*: An orchid with an extremely small population in China. Global Ecology and Conservation, 3: 367-378. doi.org/10.1016/j.gecco.2015.01.002

CITES 2021. Appendices I, II and III valid from 14 February 2021. https://cites.org/eng/app/appendices.php. Accessed 18 November 2021.

Cribb P. 1998. The Genus *Paphiopedilum*. 2nd edition. National History Publications, Borneo, Malaysia. Pp: 48-396.

Dearnaley JDW. 2007. Further advances in orchid mycorrhizal research. Mycorrhiza 17: 475-486. doi.org/10.1007/s00572-007-0138-1

Deb CR and Jakha HY. 2019. *In vitro* immature embryo culture and propagation of *Paphiopedilum villosum* Var. Boxallii (Rchb. f.) Pfitzer. Biotechnology 18: 77-88.

Deb CR and Jakha HY. 2020. Factors affecting asymbiotic immature seed culture and *in vitro* propagation of *Paphiopedilum insigne* (Wall. ex. Lindl.) Pfitzer – A horticultural important vulnerable orchid. Plant Cell Biotechnology and Molecular Biology 21: 129-141.

Deb CR and Pongener A. 2010. Search of alternative substratum for agar in plant tissue culture. Current Science, 98: 99-102.

Deb CR and Pongener A. 2013. A study on the use of low cost substrata against agar for non-symbiotic seed culture of *Cymbidium iridioides* D. Don. Australian Journal of Crop Science 7: 642-649.

Deb CR and Pongener A. 2022. Use of low cost agar alternative for *in vitro* propagation of commercially viable orchids is an attractive way for commercialization. South African Journal of Botany 150: 789-796. doi: 10.1016/j.sajb.2022.08.028

Deb CR, Pongener A and Kikon ZP. 2022. Asymbiotic immature seed culture of *Vanda bicolor* Griff., plant regeneration and formulation of an efficient new hardening technique for direct field transfer of regenerates. South African Journal of Botany 150: 744-751. doi.org/10.1016/j.sajb.2022.08.031

Diengdoh RV, Kumaria S, Tandon P and Das MC. 2017. Asymbiotic germination and seed storage of *Paphiopedilum insigne*, an endangered lady's slipper orchid. South African Journal of Botany 112: 215-224. doi: 10.1016/j.sajb.2017.05.028

Ebile PA, Opata J and Hegele S. 2022. Evaluating suitable low-cost agar substitutes, clarity, stability, and toxicity for resource-poor countries' tissue culture media. *In Vitro* Cellular & Developmental Biology – Plant 58: 989-1001. doi:10.1007/s11627-022-10285-6

Evans A and Jacquemyn H. 2022. Range size and niche breadth as predictors of climate-induced habitat change in *Epipactis* (Orchidaceae). Frontiers in Ecology and Evolution 10: 894-616. doi: 10.3389/fevo.2022.894616

Favre-Godal Q, Gourguillon L, Lordel-Madeleine S, Gindro K and Choisy P. 2020. Orchids and their mycorrhizal fungi: an insufficiently explored relationship. Mycorrhiza 30: 5-22. doi.org/10.1007/s00572-020-00934-2

Fay MF. 2018. Orchid conservation: how can we meet the challenges in the twenty-first century? Botanical Studies 59: 16. doi.org/10.1186/s40529-018-0232-z

Han J, Qiu W, Tiwari S, Bhargava R, Gaoc W and Xing B. 2015. Consumer-grade polyurethane foam functions as a large and selective absorption sink for bisphenol A in aqueous media. Journal of Materials Chemistry A 3: 8870. doi: 10.1039/c5ta00868a

Hong PI, Chen JT and Chang WC. 2008. Plant regeneration via protocorm like body formation and shoot multiplication from seed derived callus of a Maudiae type slipper orchid. Acta Physiologiae Plantarum 30: 755-759. doi:10.1007/s11738-008-0158-2

Hossain MM, Sharma M and Pathak P. 2013. *In vitro* propagation of *Dendrobium aphyllum* (Orchidaceae) – seed germination to flowering. Journal of Plant Biochemistry and Biotechnology 22: 157-167. doi: 10.1007/s13562-012-0124-3

IUCN. 2017. The IUCN red list of threatened species. 2017-2. https://www.iucnredlist.org. Accessed 18 November 2021.

Kaur S and Bhutani KK. 2016. Asymbiotic seed germination and multiplication of an endangered orchid – *Paphiopedilum venustum* (Wall. ex Sims.) Pfitz. Acta Societatis Botanicorum Poloniae 85: 34-94. doi: 10.5586/asbp.3494

Kauth P, Dutra D and Johnson T. 2008. Techniques and applications of *in vitro* orchid seed germination. In: Teixeira da Silva JA. (Eds.), *Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues*. Global Science Books, Iselworth, UK. Pp. 375-391.

Kawka M, Bubko I, Koronkiewicz M, Gruber-Bzura B, Graikou K, Chinou I, Jeziorek M, Pietrosiuk A, Sykłowska-Baranek K. 2022. Polyurethane foam rafts supported *in vitro* cultures of *Rinderagraeca* roots for enhanced production of Rinderol, potent proapoptotic naphthoquinone compound. International Journal of Molecular Science 23: 56. doi.10.3390/ijms23010056

Kolanowska M, Rewicz A and Baranow P. 2020. Ecological niche modeling of the pantropical orchid Polystachyaconcreta (Orchidaceae) and its response to climate change. Scientific Reports 10: 14801. doi.10.1038/s41598-020-71732-1

Lee YI, Yeung EC, Lee N and Chung MC. 2006. Embryo development in the lady's slipper orchid, *Paphiopedilum delenatii*, with emphasis on the ultrastructure of the suspensor. Annals of Botany 98: 1311-1319. doi:10.1093/aob/mcl222

Lee YI. 2007. The asymbiotic seed germination of six *Paphiopedilum* species in relation to the time of seed collection and seed pretreatment. Acta Horticulturae 755: 381–3865. doi 10.17660/ActaHortic.2007.755.50

Long B, Niemiera AX, Cheng ZY and Long CL. 2010. *In vitro* propagation of four threatened *Paphiopedilum* species (Orchidaceae). Plant Cell Tissue Organ Culture 101: 151-162. doi: 10.1007/s11240-010-9672-1

Longchar TB and Deb CR. 2022. Optimization of *in vitro* propagation protocol of *Dendrobium heterocarpum* Wall. ex. Lindl. and clonal genetic fidelity assessment of the regenerates: An orchid of horticultural and medicinal importance. South African Journal of Botany 149: 67-78. doi.org/10.1016/j.sajb.2022.05.058

Mao AA and Ranyaphi RA. 2013. Studies on *in vitro* asymbiotic germination of *Pahiopedilum* species of North East India. The Journal of the Orchid Society India 27: 9-13.

Martín-Forés I, Samantha B, Ben S and Greg G. 2022. Simultaneous effect of habitat remnancy, exotic species, and anthropogenic disturbance on orchid diversity in South Australia. Conservation Science and Practice 4:e12652. doi: 10.1111/csp2.12652.

Mohanty P, Paul S, Das MC, Kumaria S and Tandon P. 2012. A simple and efficient protocol for the mass propagation of *Cymbidium mastersii*: an ornamental orchid of Northeast India. AoB Plants 2012: pls023. doi:10.1093/aobpla/pls023

Pant B, Chand K, Paudel MR, Joshi PR, Thapa BB, Park SY, Shakya S, Thakuri LS, Rajbahak S, Sah AK, Baniya MK, Gurung PR, Maharjan L and Rajbhandari P. 2022. Micropropagation, antioxidant and anticancer activity of pineapple orchid: *Dendrobium densiflorum* Lindl. Journal of Plant Biochemistry and Biotechnology 31: 399-409. doi: 10.1007/s13562-021-00692-y

Paul S, Kumaria S, Tandon P. 2012. An effective nutrient medium for asymbiotic seed germination and large-scale *in vitro* regeneration of *Dendrobium hookerianum*, a threatened orchid of Northeast India. AoB Plants 2012: plr032. doi:10.1093/aobpla/plr032

Pierik RLM, Sprenkels PA, van der Harst B and van der Meys QG. 1988. Seed germination and further development of plantlets of *Paphiopedilum ciliolare* Pfitz. *in vitro*. Scientia Horticulturae 34: 139-153. doi:10.1016/0304-4238(88)90084-2

Pongener A and Deb CR. 2019. *In vitro* seed germination and propagation of *Thunia marshalliana* Rchb. f. on substrata of low cost options. Plant Cell Biotechnology and Molecular Biology 20: 204-211.

Rankou H and Kumar P. 2015. *Paphiopedilum fairrieanum*. The IUCN red list of threatened species 2015: e.T43320321A43327829. doi: 10.2305/IUCN.UK.2015-2.RLTS. T43320321A43327829.en

Rao AN. 2006. Variability identification of *Paphiopedilum*, *Anthigonium* and some vandaceous species (orchidaceae genotypes- their maintenance and propagation technology. Bulletin of Arunachal Forest Research 22: 45-51.

Rao AN. 2018. Orchid diversity of North East India: Classification, analysis and conservation issues. In: Das AP and Bera S (Eds.), *Plant Diversity in Himalaya Hotspot Region*, Vol. II. Bishen Singh Mahendra Pal Singh, Dehra Dun, India. pp. 821-863.

Roy AR, Patel RS, Patel VV, Sajeev S and Deka BC. 2011. Asymbiotic seed germination, mass propagation and seedling development of *Vanda coerulea* Griff ex. Lindl. (Blue Vanda): An *in vitro* protocol for an endangered orchid. Scientia Horticulturae128: 325-331. doi.org/10.1016/j.scienta.2011.01.023

Teixeira da Silva JA, Tsavkelova EA, Ng TB, Parthibhan S, Cardoso JC, Rao MV and Zeng SJ. 2015. Asymbiotic *in vitro* seed propagation of *Dendrobium*. Plant Cell Reports 34: 1685-1706. doi 10.1007/s00299-015-1829-2

Xu X, Fang L, Li L, Ma G, Wu K and Zeng S. 2020. Abscisic Acid inhibits asymbiotic germination of immature seeds of *Paphiopedilum armeniacum*. International Journal of Molecular Science 21: 95-61. doi:10.3390/ijms21249561

Yao L, Huang J and Zhang S. 2021. An improved protocol for asymbiotic seed germination and seedling development of *Paphiopedilum tigrinum*. Horticulturae 7: 298. doi: 10.3390/horticulturae7090298

Zeng S, Huang W, Wu K, Zhang J, Teixeirada Silva JA,Zhang J, Duan J and Duan J. 2016. *In vitro* propagation of *Paphiopedilum* orchids. Critical Reviews in Biotechnology 36: 521-534. doi: 10.3109/07388551.2014.993585

Zeng SJ, Wu KL, Teixeira da Silva JA, Zhang J, Chen Z, Xia N and Duan J. 2012. Asymbiotic seed germination, seedling development and reintroduction of *Paphiopedilum wardii* Sumerh., an endangered terrestrial orchid. Scientia Horticulturae 138: 198-209. doi:10.1016/j.scienta.2012.02.026

Zeng SJ, Zhang Y, Teixeira da Silva JA, Wu K.L, Zhang J and Duan J. 2014. Seed biology and *in vitro* seed germination of *Cypripedium*. Critical Reviews in Biotechnology 34: 358-371. doi: 10.3109/07388551.2013.841117.