J. Orchid Soc. India, 39: 95-104, 2025
https://doi.org/10.64873/JOSI.v39.i1-2. 95-104
ISSN 0971-5371

IN VITRO ASYMBIOTIC SEED GERMINATION OF A MEDICINALLY IMPORTANT AND AN ENDANGERED ORCHID, *AERIDES MULTIFLORA* ROXB.: A COMPARATIVE STUDY ON TWO NUTRIENT MEDIA

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Abstract

Aerides multiflora Roxb. is an epiphytic orchid with significant medicinal and ornamental value. The present study was aimed to standardize mass multiplication through *in vitro* asymbiotic seed germination on M and MS nutrient media, with and without growth additives. The investigation focused on evaluating the effects of different combinations of growth regulators on seed germination, protocorm formation and their subsequent development into seedlings. The highest germination rate (98.75%) was achieved on M medium supplemented with kinetin (KN) and activated charcoal (AC). Furthermore, M medium enriched with IBA (0.5 mgL^{-1}) proved suitable for seed germination, protocorm multiplication, and development of seedlings. The standardized protocol offers an efficient method for the large-scale propagation of *A. multiflora* and provides a potential strategy to reduce commercial collection pressures on natural populations of this species.

Introduction

ORCHIDS ARE renowned for the complexity and captivating beauty of their highly colourful, long-lasting flowers, which display remarkable diversity in shape and size, making them the doyens among ornamentally important plants. The applications of tissue culture techniques has further enhanced their value, opening new avenues in plant breeding and propagation. These methods enable mass multiplication of superior genotypes for cut flower production and allow the establishment of plants within a significantly shortened time frame. Orchidaceae, as one of the largest families of flowering plants, comprises 29,481 accepted species worldwide (WFO, 2023). In India, the family is represented by 1,256 species under 155 genera (Singh *et al.,* 2019). Beyond their ornamental significance, orchids also hold considerable therapeutic value, serving as a source of bioactive compounds with applications against various ailments. Moreover, herbs and herbal formulations continue to play a vital role in primary healthcare systems across many regions of the world (Jawahar *et al*., 2008; Pathak *et al.,* 2010). Traditional Indian literature on medicinal plants provides significant insights into the therapeutic value of orchids (Hossain, 2011; Pathak *et al*., 2010; Tikendra *et al*., 2020). Orchids are distributed across nearly all regions of the world with their highest diversity in tropical and subtropical areas, excluding Antarctica and arid desert ecosystems.

Aerides multiflora Roxb. is a monopodial, aromatic epiphytic orchid that typically grows on tree trunks with an erect stem, 5-7 cm in length and 10-15 mm in thickness, its base is sheathed with the persistent remains of fallen leaf bases. From the base, emerges a dense cluster of roots, each 2-3 mm thick, clothed in a well-developed velamen that ensures efficient anchorage and absorption. The species is indigenous to Bangladesh, Cambodia, India, Laos, Malaysia, Myanmar, Nepal, Philippines, Thailand, and Vietnam. In India, it is geographically distributed over NorthWestern Himalayas, Sikkim, West Bengal, and Arunachal Pradesh (Singh *et al.,* 2019). The plant is commonly used for treating wounds, bacterial infections, and possess anti-diabetic property (Sharma and Pathak, 2024; Thapa *et al*., 2022). Root paste is used for curing arthritis, rheumatism and also used against cuts and wounds; leaf juice is used for nasal congestion; and the powder of tuber is used for healing wounds and boils and also for chronic cold and cough (Singh and Duggal, 2009). The species has been listed under Appendix II of CITES as it has been illegally harvested and traded in for its use in local traditional medicine, horticulture, and international trade (CITES, 2025). However, the size and frequency of its natural populations are on a gradual decline because of habitat destruction, and unscrupulous collections for commercial and scientific purposes. Conventional conservation practices alone are insufficient to prevent the overexploitation of this medicinal orchid in its natural habitat. Tissue culture and micropropagation techniques provide an effective alternative, offering the possibility of large-scale multiplication within a short period. Establishing a reliable *in vitro* propagation protocol is therefore essential, both to support *ex situ* conservation and to reduce pressure on natural populations of

Received: July 9, 2025; Accepted: September 30, 2025

commercially important and/or endangered orchids. As seed germination, protocorm formation, and seedling/ plantlet development in orchids are influenced by factors such as light, temperature, developmental stage of explants, and plant growth regulators (PGRs), systematic studies are required to optimize culture conditions for different species in orchids (Arditti *et al.*, 1982a,b; Dhillon *et al.*, 2023; Pathak *et al.*, 2001). Hence, the present study was planned with a view to developing an efficient an *in vitro* asymbiotic seed germination protocol for *A. multiflora*, with an aim of contributing to its conservation and sustainable utilization.

Material and Methods

Sample Collection and Experimental Site

Immature seeds procured from green unripe capsules (16 wap) were collected in the month of July from Sarkaghat, Mandi, Himachal Pradesh, India. The experiments were carried out in the Orchid laboratory, Department of Botany, Panjab University, Chandigarh.

Seed Viability Test

Seed viability was determined by the ability to reduce 2,3,5- triphenyltetrazolium chloride (TTC) to the red coloured formazan (Brewer, 1949). The orchid embryos either turned red or stayed colourless after TTC staining. Seeds with TTC reduction ability (red coloured) were scored as viable as evidenced from microscopic examination.

Culture Media and Culture Conditions

In the present study, two different nutrient media [Mitra *et al.* (M, 1976) and Murashige and Skoog (MS, 1962),] used for seed germination and seedlings development were augmented with sucrose (2%; Daurala Sugar works, Uttar Pradesh), agar powder (0.8%; Himedia, Mumbai) and different growth additives. The growth regulators [auxins: Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA) and cytokinins: Kinetin (KN), 6-Benzylamino-purine (BAP)] in various combinations and 0.5 mgL^{-1} concentration were used (Table 1 and 2). Activated Charcoal (AC; 2 gL^{-1}; Thermo Fisher Scientific, Mumbai) was also used in one set of experiments. The pH of the nutrient media was adjusted to 5.7-5.8 with either HCl or 0.1 N NaOH. The test tubes containing nutrient media were autoclaved at 121°C with 15 psi pressure for 20 min.

Sterilization of the Explant and Culture Conditions

Green capsules were washed in running tap water using Teepol (detergent) for 20 min and then, rinsed with distilled water. The washed capsules were taken inside a laminar air flow hood where these were treated with Bavistin (0.01%; 7-8 min), Streptomycin (0.01%; 5-6 min), and Mercuric Chloride (0.1%; 3-4 min). After each treatment, capsules were rinsed thrice with autoclaved distilled water. The capsules were flame sterilized with ethyl alcohol (70%) for 2-3 sec and then, left on a sterile filter paper in a sterile petriplate to absorb excess water. The immature capsules were then split open vertically with sterile surgical blade and the powdery seeds were inoculated on nutrient medium in the test tubes, each containing 25 ml medium. The cultures were maintained under a 12 hrs photoperiod of 3500 lux light intensity and a temperature of 25±2°C and observed regularly.

Data Recordings and Statistical Analysis

The experiment was conducted using 4 replicates per treatment. The cultures were examined at regular intervals and different parameters such as days required for onset of germination, spherule and protocorm development, first leaf and root primordia development, complete seedling formation and seed germination percentage were recorded. Data obtained from the present investigation were subjected to analysis of variance (ANOVA) and significant differences were determined by employing Tukey's test at $p<0.05$. The statistical data analysis was performed using SPSS (version 16) software. The results are presented as mean±standard error of both the experiments. Seed germination percentage was derived using the following formula:

$$\text{Seed germination percentage} = \frac{\text{(Number of seeds successfully germinated by swelling)}}{\text{(Total number of seeds inoculated)}} \times 100$$

Acclimatization

Healthy seedlings with 2-3 well grown leaves and 1-2 roots were gradually hardened *in vitro*, by sequential elimination of growth additives, vitamins, sucrose, and minor salts from the nutrient matrix at 15 days interval. The well rooted seedlings were taken out from culture vessels and thoroughly washed under running tap water for removal of agar attached to root surface and transferred to pots containing a potting mixture of brick pieces, *Sphagnum* moss, charcoal pieces, and coconut husk in 1:1:1:1 ratio.

Results and Discussion

In the present investigation, the immature seeds of *A. multiflora* obtained from the unopened green capsules (Fig. 1a-b) were successfully used as explants (Fig. 1c). Prior to inoculation, the seed viability was determined by TTC (2,3,5-triphenyl tetrazolium chloride)

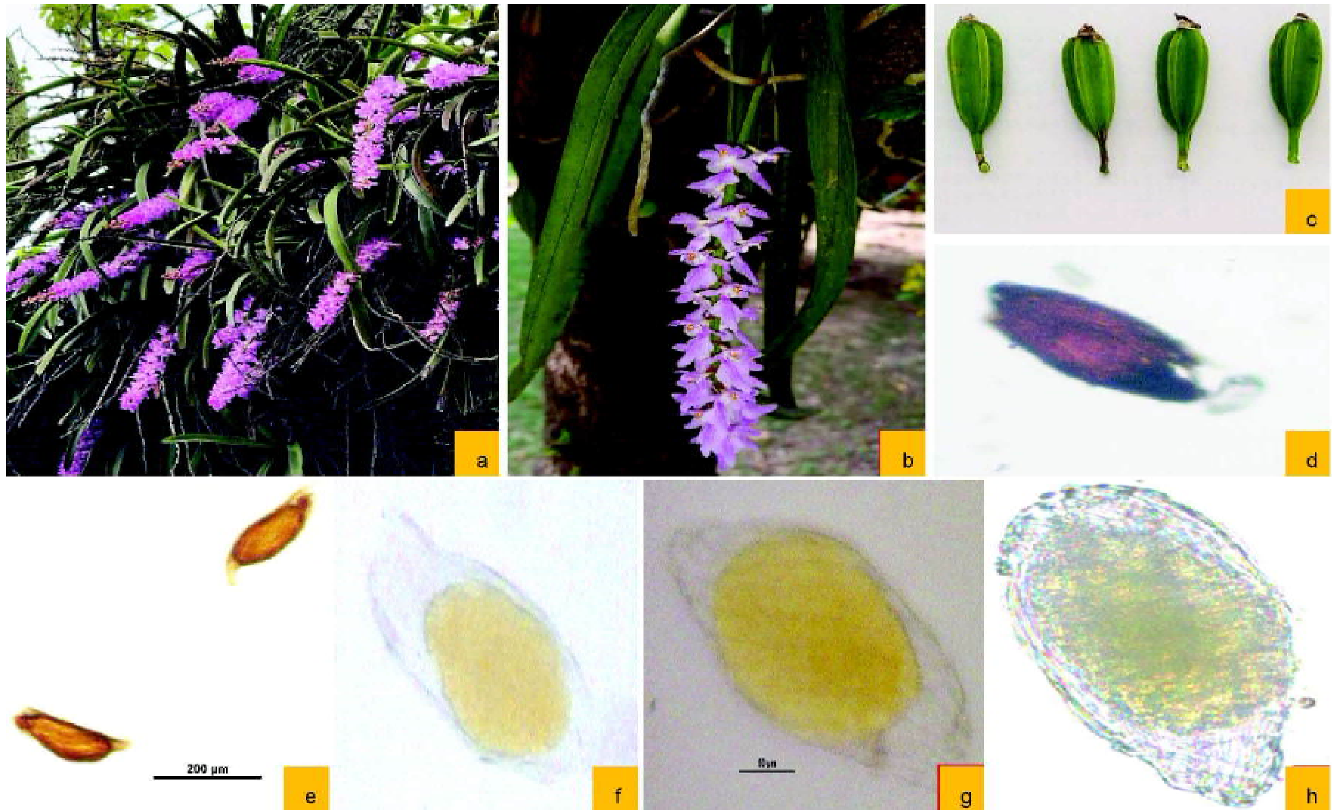

Fig. 1a-h. *In vitro* asymbiotic seed germination and seedling development in ***Aerides multiflora***: a, Plant in full bloom; b, Magnified view of the inflorescence; c, Immature capsules; d, TTC testing (×10); e, Seeds at the time of inoculation; f-g, Swelling of embryo; h, Rupturing of seed coat and emergence of spherule.

test as the seeds have their ability to reduce TTC to red coloured formazan. Seeds were found to be viable, as determined by positive formazan staining, and morphologically 98% seeds appeared normal with viable embryos (Fig. 1d). Due to the transparency of the seed coat, developmental changes during culture could be directly monitored under the microscope. Seed germination was characterized by the swelling of the embryo until it reached the width of the seed coat, followed by the emergence of the embryo (Fig. 1f-h). Once the embryo was completely discharged from seed coat, it formed a structure referred to as a spherule. This stage was succeeded by the development of protocorms (Fig. 2a), which subsequently differentiated first leaf and root primordia, and healthy seedlings. The duration of *in vitro* germination was influenced by factors such as the nutrient medium employed, the type of growth additives employed, and the species under investigation.

The seeds of the presently studied species successfully germinated in both the nutrient media used. Highest seed germination frequency (92.75±0.47%) and early seedling formation (120.00±0.40 days) were observed on M followed by MS (89.66±0.33%; 135.33±0.33days) nutrient media (Figs. 1-2; Tables 1-2). The efficacy of *in vitro* asymbiotic seed germination for orchid mass propagation has been validated through some studies, encompassing a broad range of species and hybrids (Anuprabha and Pathak, 2012; Arditti *et al*., 1982; Basu *et al*., 2024; Bhowmik and Rahman, 2020, 2022, 2023; Dhillon and Pathak, 2023; Gangaprasad *et al.,* 2024; Giri and Tamta, 2012; Hyeong *et al*., 2023; Jaryal *et al*., 2025; Kirti *et al*., 2023; Lekshmi and Decruse, 2018; Pathak *et al*., 1992, 1999, 2001, 2007, 2011; 2016, 2023; Park *et al*., 2023; Sunita *et al*., 2021; Thakur and Pathak, 2020, 2021; Vasundhra *et al*., 2021; Vij and Pathak, 1988; Vij *et al*., 1995; Zeng *et al*., 2012). Incorporation of AC (2 gL^{-1}) in M and MS media proved beneficial for enhancing the germination frequency (97.75±0.25% and 97.66±0.33%, respectively). The beneficial effect of AC on *in vitro* seedling growth has also been earlier documented by a few researchers (Druart and Wulf, 1993; Fridborg and Eriksson, 1975; Ket *et al*., 2004; Pathak *et al*., 2001; Thakur and Pathak, 2021; Thomas and Michael, 2007). Its inclusion in the culture medium proved advantageous, as AC adsorbs growth-inhibiting compounds such as polyphenols, which can negatively affect plant development (Fridborg and Eriksson, 1975). Pierik *et al.* (1988) further noted that AC is not essential during the initial stages of seedling growth, it plays a crucial role in subsequent developmental stages.

Exogenous growth regulators are generally recommended for initiating and maintaining orchid cultures, though their effectiveness largely depends on both type and concentration (Arditti *et al.*, 1982; Pathak *et al.*, 2001). Hayes (1969) reported that orchid seeds possess relatively low levels of endogenous auxins, and

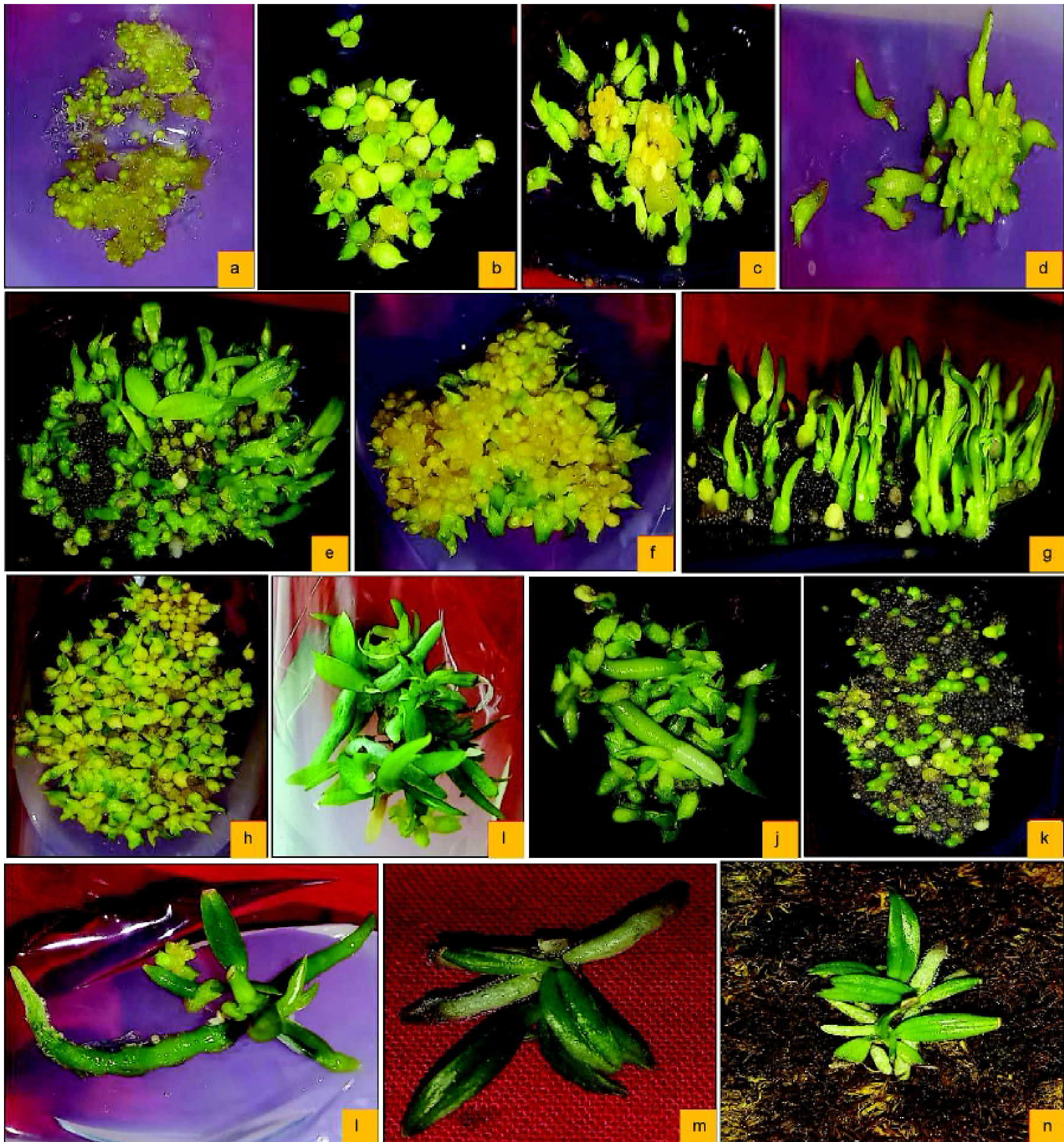

Fig. 2a-n. *In vitro* asymbiotic seed germination and seedling development in ***Aerides multiflora*** (contd.): a, Protocorm development and seed callus formation (M); b, Formation of globular and healthy protocorms (M+AC); c, Protocorms multiplication via callusing (M+IBA+AC); d, Leaf differentiation (MS+IBA); e, Leaf formation and protocorms browning (MS+IAA+AC); f, Rapid multiplication of protocorms (M+BAP); g, Emergence of healthy shoots (MS+IBA+AC); h, Protocorms multiplication and emergence of leaf primordia (M+IAA+KN); i, Healthy seedlings (MS+IAA+BAP); j, Healthy seedlings with elongated roots (M+KN+AC); k, Emergence of leaf primordia and browning of protocorms (MS+IBA+AC); l, Healthy seedlings with elongated roots (MS+IBA+BAP); m, Complete healthy seedling with 4-5 leaves and 2-3 roots; n, Seedlings transferred to a pot containing potting mixture of charcoal pieces, coconut husk, brick pieces and ***Sphagnum*** moss (1:1:1:1).

Table 1. *In vitro* immature seed (16 wap) germination and seedling development on Mitra *et al*. (1976) medium, in *A. multiflora*.

Growth additives	Germination frequency(%)	Time taken in days						Remarks
		Onset of germination	Spherule formation	Protocorm formation	1st Leaf primordia	1st root primordia	Complete seedling	
-	92.75±0.47	18.00±0.41^{g}	23.75±0.62^{f}	30.00±0.40^{e}	53.75±0.95^{ef}	80.00±0.70g^{h}	120.00±0.40^{h}	Protocorms multiplication via callusing
AC	97.75±0.25	13.75±0.25^{def}	19.50±0.28cd	25.00±0.00^{cd}	46.50±0.28^{b}	78.25±0.25^{fg}	115.75±0.85^{ef}	Healthy seedlings; Large sized globular protocorms
IAA	98.25±0.25	12.75±0.94^{de}	18.75±0.47^{cd}	24.50±0.50^{c}	51.50±0.50^{de}	73.00±0.71^{d}	109.25±0.47^{d}	Rapid multiplication of protocorms via callusing
IAA+AC	97.50±0.28	11.75±0.25^{cd}	17.50±0.28^{bc}	22.75±0.47^{bc}	47.75±0.47^{b}	71.50±0.28^{cd}	106.75±0.25^{c}	Healthy seedlings
IBA	97.50±0.64	8.75±0.47^{ab}	13.50±0.28^{a}	23.00±0.91^{bc}	46.00±0.70^{b}	68.75±0.48^{ab}	95.75±0.47^{b}	Rapid multiplication of protocorms via callusing
IBA+AC	96.50±0.28	8.25±0.25^{a}	13.00±0.57^{a}	19.50±0.28^{a}	43.25±0.25^{a}	66.75±0.48^{a}	92.25±0.25^{a}	Protocorms multiplication via callusing
KN	97.75±0.25	15.50±0.29^{fg}	22.50±0.28^{ef}	30.50±0.64^{e}	52.50±0.28^{de}	80.50±0.50^{h}	118.50±0.28^{gh}	Protocorm multiplication; Healthy seedlings
KN+AC	98.75±0.57	13.00±0.00^{de}	19.50±0.29^{cd}	27.00±0.40^{d}	50.50±0.28^{cd}	75.75±0.47^{e}	114.50±0.50^{e}	Healthy seedlings with elongated roots
BAP	98.00±0.25	19.50±0.28^{hi}	24.50±0.29^{fg}	32.00±0.57^{e}	56.50±0.50^{g}	81.75±0.25^{hi}	119.25±0.25^{h}	Rapid multiplication of protocorms via callusing
BAP+AC	97.75±0.41	17.50±0.28^{gh}	23.75±0.48^{f}	30.50±0.28^{e}	53.50±0.28^{ef}	80.75±0.48^{h}	117.75±0.25^{fgh}	Healthy seedlings
IAA+KN	97.00±0.47	10.50±0.29^{bc}	16.25±0.25^{b}	21.50±0.28^{ab}	48.25±0.25^{bc}	69.50±0.29b^{c}	105.25±0.25^{c}	Multiplication of protocorms; Healthy seedlings
IAA+BAP	97.75±0.47	14.50±0.28^{ef}	20.50±0.28^{de}	27.00±0.58^{d}	55.75±0.47^{fg}	76.50±0.29^{ef}	116.50±0.64^{efg}	Rapid protocorms multiplication via callusing; Healthy seedlings
IBA+KN	98.25±0.40	21.25±0.62^{i}	26.50±0.64^{g}	31.00±0.41^{e}	60.50±0.28^{h}	85.00±0.41^{j}	133.75±0.62^{j}	Healthy seedlings
IBA+BAP	95.00±0.41	19.50±0.29^{hi}	24.50±0.28^{fg}	30.00±0.41^{e}	59.50±0.28^{h}	83.75±0.25^{ij}	130.75±0.47^{i}	Healthy seedlings

Entries in column number 2 to 7 are Mean ± S.E.; same alphabetical letter in the superscript denotes that the corresponding mean are in the same group using Tuckey test at 5%.

Table 2. *In vitro* immature seed (16 wap) germination and seedling development on Murashige and Skoog, 1962 (MS) medium, in *A. multiflora*.

Growth additives	Germination frequency (%)	Time taken in days						Remarks
		Onset of germination	Spherule formation	Protocorm formation	1st Leaf primordia	1st root primordia	Complete seedling	
-	89.66±0.33	26.33±0.66^{i}	31.66±0.33^{fg}	38.66±0.33^{fgh}	64.66±0.33^{h}	88.66±0.33^{fg}	135.33±0.33^{jk}	Protocorms multiplication via callusing
AC	91.66±0.33	21.33±0.33efg	29.33±0.33^{d}	35.33±0.33^{cd}	60.33±0.33^{def}	83.66±0.33^{e}	129.33±0.33^{hi}	Rapid multiplication of protocorms via callusing; Seed callusing; Seedling with broad leaves
IAA	97.66±0.33	15.66±0.33^{b}	20.33±0.33^{a}	26.33±0.33^{a}	48.66±0.33^{b}	71.00±0.58^{b}	103.33±0.33^{b}	Rapid multiplication of protocorms via callusing; Healthy seedlings
IAA+AC	95.33±0.33	13.67±0.33a	20.67±0.33^{a}	27.00±0.00^{a}	45.67±0.33^{a}	68.67±0.33^{a}	98.67±0.67^{a}	Protocorms multiplication
IBA	95.00±0.00	19.66±0.33^{cde}	29.33±0.66^{de}	31.33±0.33^{b}	59.00±1.00^{de}	79.00±0.58^{d}	119.67±0.33^{f}	Healthy seedlings
IBA +AC	96.67±0.33	19.00±0.00^{cd}	27.66±0.67^{cde}	37.00±0.57^{defg}	55.00±0.58^{c}	76.33±0.33^{c}	115.33 ±0.33^{e}	Healthy seedlings
KN	94.33±0.33	21.66±0.33^{fg}	28.66±0.66^{de}	34.33±0.67^{c}	63.00±0.00^{gh}	90.66±0.67^{gh}	127.33±0.33^{h}	Protocorms multiplication
KN +AC	94.67±0.67	19.33±0.33^{cd}	28.66±0.33^{de}	36.33±0.33^{de}	59.67±0.33^{de}	87.67±0.33^{f}	122.67±0.67^{g}	Protocorms multiplication; Healthy seedlings
BAP	94.00±0.00	20.33±0.67^{def}	26.00±0.00^{c}	36.66±0.33^{def}	61.33±0.67^{efg}	84.66±0.33^{e}	111.67±0.33^{d}	Protocorms multiplication via callusing
BAP +AC	96.33±0.33	19.67±0.33^{cde}	27.33±0.33cd	37.67±0.33^{efgh}	58.33±0.33d	79.67±0.33d	109.00±0.58c	Healthy seedlings
IAA+KN	95.66±0.33	18.33±0.33^{c}	23.66±0.33^{b}	33.66±0.33^{c}	53.66±0.67^{c}	84.33±0.33^{e}	114.67±0.33^{e}	Protocorms multiplication via callusing
IAA+BAP	92.66±0.33	22.33±0.33^{gh}	29.66±0.33^{ef}	39.00±0.58^{gh}	58.33±0.33d	89.66±0.33^{fg}	124.33±0.66^{g}	Healthy seedlings
IBA+KN	92.33±0.33	24.00±0.00^{h}	32.00±0.00^{g}	41.33±0.33^{i}	62.33±0.33^{fgh}	93.66±0.67^{i}	132.33±0.33^{j}	Protocorms multiplication via callusing
IBA+BAP	93.66±0.67	22.33±0.33^{gh}	29.66±0.33^{ef}	39.66±0.33^{hi}	63.33±0.33^{gh}	92.33±0.33^{hi}	130.67±0.33^{ij}	Healthy seedlings

Entries in column number 2 to 7 are Mean ± S.E.; same alphabetical letter in the superscript denotes that the corresponding mean are in the same group using Tuckey test at 5%.

in nature, their germination is supported by mycorrhizal associations that supplement the auxin deficiency. Addition of IBA in the both M and MS media proved beneficial for advancing the onset of germination (8.75±0.47 days; 19.66±0.33 days), spherule formation (13.50±0.28 days; 29.33±0.66 days), protocorm development (23.00±0.91 days; 31.33±0.33 days), and differentiation of first leaf (46.00±0.70 days; 59.00±1.00 days) and root (68.75±0.48 days; 79.00±0.58 days) primordia, respectively. Chauhan *et al.* (2010) recommended M medium supplemented with IBA as the most effective for early differentiation and subsequent seedling development. Promotory effects of IBA have previously been reported during seed germination in *Acampe papillosa* (Vij and Malhotra, 1988), *Bulbophyllum careyanum* and *Dendrobium chrysotoxum* (Kher, 1999), as well as in *Cypripedium* and *Vanilla planifolia* (Hegarty, 1955). Similar positive responses were also observed for protocorm development and multiplication in *Coelogyne cristata* (Vasundhra, 2023), *Cymbidium aloifolium* (Vasundhra, 2023), *Dendrobium* (Pages, 1971) and for seedling development of *Eulophia dabia* (Sharma and Vij, 1986). KN in the M medium slightly increased the germination response (97.75±0.25%), but this combination proved ineffective for advancing all the morphogenetic stages. However, supplementation of KN in the MS medium proved effective during early stages of seed germination and induced protocorm multiplication via callusing. KN has been reported to stimulate protocorm proliferation in several orchids, including *Cymbidium eburneum*, *Aerides multiflora*, *Eria spicata*, and *Pholidata articulata* (Mahant, 1991; Pathak, 1989). This cytokinin has also been reported to enhance germination in several other orchid species, including *Coelogyne fimbriata* (Anuprabha and Pathak, 2020), *Dendrobium chrysotoxum* (Bhowmik and Rahman, 2020), *Rhynchostylis retusa* (Vasundhra, 2023), *Vanda cristata* (Pathak *et al*., 2023), *V. tessellata* (Madhavi and Shankar, 2019), and *V. testacea* (Kaur, 2021).

When IAA and KN was used together in M medium, synergistic effect was apparent in the form of early initiation of seed germination (10.50±0.29 days), spherule formation (16.25±0.25 days), growth and multiplication of protocorms (21.50±0.28 days; Fig. 2h), differentiation of first leaf (48.25±0.25 days) and root (69.50±0.28 days) primordia and healthy seedlings development (105.25±0.25 days). Interestingly, the benign effect of IAA was masked in the presence of KN, when the same nutritional combination was incorporated to MS medium. However, multiplication of protocorms and seed callusing was observed in this combination. Similar promontory responses of this combination have been reported earlier by Verma (2016) in *Satyrium nepalense* including higher germination frequency, accelerated rhizogenesis, and improved seedling development. Tikendra *et al.* (2018) observed a significant increase in shoot and root formation in *Dendrobium thyrsiflorum* when M medium was supplemented with IAA (1.0 mgL^{-1}) and KN (1.0 mgL^{-1}). Conversely, the combined presence of IAA and KN in the nutrient medium has been reported to reduce seed germination earlier in *Aerides multiflora*, *Eria spicata*, *Vanda cristata* (Pathak, 1989), and *Dactylorhiza* (Hadley, 1970). Based on the present results, M medium supplemented with IBA (0.5 mgL^{-1}) is suggested as the optimal nutritional combination during the seed germination, growth and development of seedlings, in *A. multiflora*.

Conclusion

Conservation priorities for orchid species remain highly challenging, as their interactions with diverse groups of organisms are differentially affected by anthropogenic pressures and environmental perturbations. In this context, *in vitro* asymbiotic seed culture technique has gained significant attention for large-scale propagation of orchids. In the present study, an efficient *in vitro* propagation protocol using the immature seeds was established for an endangered and medicinally important orchid, *A. multiflora*, which may serve as a model for similar studies in related taxa. The results highlighted the crucial role of exogenous plant growth regulators for supporting the *in vitro* asymbiotic seed germination and growth and development of orchid seedlings.

References

Anuprabha and Promila Pathak. 2012. Green pod culture in *Dendrobium chrysanthum* Lindl. A study *in vitro*. *J. Orchid Soc. India*, **26**(1-2): 105-09.

Anuprabha and Promila Pathak. 2020. Micropropagation of *Coelogyne fimbriata* Lindl. using pseudobulb explants. *J. Orchid Soc. India,* **34**: 131-36.

Arditti, J., J. D. Michaud, and A. P. Oliva. 1982a. Practical germination of North American and related orchids-I. *Epipactis atrorubens, E. gigantea* and *E. helleborine*. *Am. Orchid Soc. Bull.*, **51**: 162-71.

Arditti, J., M. A. Clements, G. Fast, G. Hadley, G. Nishimura, and R. Ernst. 1982b. Orchid seed germination and seedling culture- A manual. *In: Orchid Biology: Reviews and Perspectives,* Vol. II. (ed. J. Arditti) pp. 243-370. Cornell University Press, Ithaca, New York, U.S.A.

Basu, S., U. Roy, and K. Mitra. 2024. *In vitro* propagation of *Coelogyne flaccida* Lindl.: An orchid having horticultural and therapeutic potential. *J. Orchid Soc. India*, **38**: 125-33.

Bhowmik, T. K. and M. M. Rahman. 2020. *In vitro* seed germination and micropropagation of *Dendrobium chrysotoxum* Lindl. (Golden Bow): A highly fragrant orchid species of Bangladesh. *J. Orchid Soc. India,* **34**: 69-77.

Bhowmik, T. K. and M. M. Rahman. 2022. Seed germination, protocorm multiplication, and seedling development in *Dendrobium formosum* Roxb. ex Lindl. of Bangladesh- A study *in vitro*. *J. Orchid Soc. India*, **36**: 1-7.

Bhowmik, T. K. and M. M. Rahman. 2023. *In vitro* seed, seedling and SPSs development in *Habenaria digitata* Lindl. on different growth additives and PGRs supplemented MS medium. *J. Orchid Soc. India*, **37**: 59-67.

Brewer, H. E. 1949. Tetrazolium chloride as a test for damage in artificially cured peanuts. *Science*, **110**: 451-52.

Chauhan, S., Promila Pathak, Sanjay Sharma, and S. P. Vij. 2010. *In vitro* asymbiotic seed germination of *Satyrium nepalense*, an endangered medicinally important orchid. *J. Orchid Soc. India*, **24**(1-2): 61-66.

CITES. 2025. *The CITES Appendices*. Available at: https://cites.org/eng/app/appendices.php. Geneva, Switzerland.

Dhillon, M. K. and Promila Pathak. 2023. Asymbiotic seed germination in a medicinally important and near threatened terrestrial orchid, *Crepidium acuminatum* (D.don) Szlach. from NorthWestern Himalayas: A study *in vitro*. *J. Orchid Soc. India,* **37**: 49-57.

Druart, P. and O. D. Wulf. 1993. Activated charcoal catalyses sucrose hydrolysis during autoclaving. *Plant Cell Tiss. Organ Cult*., **32:** 97-99.

Fridborg, G. and T. Eriksson. 1975. Effects of activated charcoal on growth and morphogenesis in cell cultures. *Physiol. Plant*, **34**: 306-08.

Gangaprasad, A., S. Jerald, P. M. Salim Pichan, and Mehtab J. Bukhari. 2024. Asymbiotic seed germination and enhanced shoot production in dual phase culture system using organic additives in *Dendrobium ovatum* (L.) Kraenzl. *J. Orchid Soc. India*, **38**: 45-54.

Giri, D. and S. Tamta. 2012. Propagation and conservation of *Dactylorhiza hatagirea* (D.Don) Soo, An endangered alpine orchid. *Afr. J. Biotechnol*., **11**(62): 12586-94.

Hadley, G. 1970. The interaction of kinetin, auxin and other factors in the development of north temperate orchids. *New Phytol*., **69**: 549-55.

Hayes, B. A. 1969. Observations on orchid seed mycorrhizae. Mycopathol. Mycol. Appl., 38: 139-44.

Hegarty, C. P. 1955. Observations on the germination of orchid seeds. *Amer. Orchid Soc. Bull*., **24**: 457-64.

Hossain, M. M. 2011. Therapeutic orchids: Traditional uses and recent advances- An overview. *Fitoterapia*, **82**: 102-40.

Hyeong, B. P., J. An, Bae Kee-Hwa, S. H. Hong, H. J. Park, S. Kim, W. L. Chang, L. Byoung-Doo, J. H. Baek, Y. K. Nam, and J. E. Hwang. 2023. Asymbiotic seed germination and *in vitro* seedling development of the endangered orchid species *Cypripedium guttatum*. *Plants,* **12**(22): 3788. https://doi.org/10.3390/plants12223788

Jaryal, P., Promila Pathak, V. Jaiswal, and A. R. Warghat. 2025. Identification of an endangered and medicinally important Himalayan orchid, *Dactylorhiza hatagirea* D.Don Soo using DNA barcodes and development of an efficient *in vitro* propagation protocol utilizing embryo culture technique. *In Vitro Cell. Dev. Biol. Plant*, 1-13. https://doi.org/10.1007/s11627-025-10556-y

Jawahar, M., S. Ravipaul, and M. Jeyaseelan. 2008. *In vitro* regeneration of *Vitex negunda* L.- A multipurpose woody aromatic medicinal shrub. *Plant Tiss. Cult. Biotechnol*., **18**: 37-42.

Kaur, S. 2021. *In vitro* propagation of *Vanda testacea* (Lindl.)Reichb.f.: A medicinally important threatened orchid. *Plant Tissue Cult. Biotechnol*., **31**(2): 153-60.

Ket, N. V., E. J. Hahn, S. Y. Park, D. Chakrabarty, and K. Y. Peak. 2004. Micropropagation of an endangered orchid, *Anoectochilus formosanus. Biol. Plant*., **48**: 339-44.

Kher, A. 1999. *In vitro Propagation of Some Economically Important Orchids with a Particular Reference to the Cytological Status of the Propagules*. Ph.D. Thesis, Panjab University, Chandigarh, India.

Kirti, Promila Pathak, and K. C. Mahant. 2023. Asymbiotic seed germination and seedling development in a commercially important and endemic orchid of Western Ghats, *Aerides crispa* Lindl.- A study *in vitro*. *J. Orchid Soc. India*, **37**: 141-49.

Lekshmi, S. and S. W. Decruse. 2018. *In vitro* symbiotic seed germination of *Vanda spathulata* (L.) Spreng., A vulnerable orchid of Western Ghats. *J. Orchid Soc. India*, **32**: 113-19.

Madhavi, M. and P. C. Shankar. 2019. Effects of different growth additives on seed germination of *Vanda tessellata* (Roxb.) Hook. ex G. Don- A medicinal orchid. *J. Orchid Soc. India,* **33**: 105-12.

Mahant, K. C. 1991. *Green Pod Culture and Regeneration Potential in Some Indian Orchids: A Study in vitro*. Ph.D. Thesis, Panjab University, Chandigarh, India.

Mitra, G. C., R. N. Prasad, and A. Roy Chowdhury. 1976. Inorganic salts and differentiation of protocorms in seed callus of an orchid and correlated changes in its free amino acid content. *Indian J. Exp. Biol*., **14**: 350-51.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco cultures. *Plant Physiol*., **15**: 473.

Pages, P. D. 1971. Banana homogenate, coconut water, peptone and auxins as nutrient supplements in the *in vitro* culture of *Dendrobium* and *Phalaenopsis* ovules. *SERCA Bull.* No. 2, College Leguna, Philippines.

Park, H. B., J. An, K.-H. Bae, S. H. Hong, H. J. Park, S. Kim, C. W. Lee, B.-D. Lee, J. H. Baek, N. Y. Kim, and J. E. Hwang. **2023.** Asymbiotic seed germination and *in vitro* seedling development of the endangered orchid species, *Cypripedium guttatum*. *Plants,* **12**: 3788. https://doi.org/10.3390/plants12223788

Pathak, Promila. 1989. *Asymbiotic Germination and clonal propagation of some commercially important and endangered orchids of India using tissue culture techniques*. Ph.D. Thesis, Panjab University, Chandigarh, India.

Pathak, Promila and S. P. Vij. 2007. On developing a cost effective protocol by using alternate cheap gelling agents during asymbiotic germination in *Cymbidium pendulum* Roxb. Sw.: A study *in vitro. In: Proc. 9th Asean Pacific Orchid Conference*, Soyang, South Korea.

Pathak, Promila, K. C. Mahant, and S. P. Vij. 1999. Green pod culture in *Herminium lanceum* Thunb. ex Sw. *In: Proc. National Seminar on Plant Genetic Diversity: Evaluation and Conservation*, pp. 43, Panjab University, Chandigarh, India.

Pathak, Promila, K. C. Mahant, and A. Gupta. 2001. *In vitro* propagation as an aid to conservation and commercialization of Indian orchids: Seed culture. *In: Orchids: Science and Commerce* (eds. Promila Pathak, R. N. Sehgal, N. Shekhar, M. Sharma, and A. Sood) pp. 319-62. Bishen Singh Mahendra Pal Singh, Dehradun, India.

Pathak, Promila, S. P. Vij, and K. C. Mahant. 1992. Ovule culture in *Goodyera biflora* (Lindl.) Hk.f.: A study *in vitro*. *J. Orchid Soc. India*, **6**: 49-53.

Pathak, Promila, Sanjeev K. Arora, Shivani Verma, Kriti Dhiman, K. C. Mahant, and Raja Jeet. 2016. Mass propagation of a floriculturally and medicinally important epiphytic orchid *Dendrobium amoenum* Wall. ex Lindl. through asymbiotic seed culture: A study *in vitro. Pb. Univ. Res. J. (Sci).*, **66**: 39-45.

Pathak, Promila, A. Bhattacharya, S. P. Vij, K. C. Mahant, Mandeep K. Dhillon, and H. Piri. 2010. An update on the medicinal orchids of Himachal Pradesh with brief notes on their habit, distribution and flowering period. *J. Non Timb. For. Prod.*, **17**(3): 365-72.

Pathak, Promila, Anamika Kumari, Brent D. Chandler, and Lawrence W. Zettler. 2023. *In vitro* propagation and phytochemical analysis of *Vanda cristata* Wall. ex Lindl: An endangered medicinal orchid of biopharmaceutical importance. *S. Afr. J. Bot.,* **153**: 109-23.

Pathak, Promila, H. Piri, S. P. Vij, K. C. Mahant, and S. Chauhan. 2011. *In vitro* propagation and mass scale multiplication of a critically endangered epiphytic orchid, *Gastrochilus calceolaris* (Buch.-Ham ex JE Sm.) D. Don using immature seeds. *Indian J. Exp. Biol.,* **49**(9): 711-16.

Pierik, R. L. M., P. A. Sprenkels, B. Van der Harst, and Q. G. Van der Meys. 1988. Seed germination and further development of *Paphiopedilum ciliolare* Pfitz *in vitro. Sci. Hortic.*, **34:** 139-53.

Sharma, S. and Promila Pathak. 2024. Elucidating the chemical composition and antioxidant activity of a therapeutically important and endangered orchid, *Aerides multiflora* roxb. from NorthWest Himalayas. *World journal of pharmacy and pharmaceutical science (WJPPS)*, **13**(10): 742-65.

Sharma, M. and S. P. Vij. 1986. Micropropagation of *Eulophia dabia* (D.Don) Hochr. *Orchid News*, **2**: 11.

Singh, A. and S. Duggal. 2009. Medicinal orchids: An overview. *Ethnobot. Leafl.*, **13**: 351-63.

Singh, S. K., D. K. Agarwala, J. S. Jalal, S. S. Dash, A. A. Mao, and Paramjit Singh. 2019. *Orchids of India: A Pictorial Guide.* BSI, Kolkata, India.

Sunita, Promila Pathak, and K. C. Mahant. 2021. Green pod culture of an endangered and medicinally important orchid, *Vanda cristata* Wall. ex Lindl. from Himachal Pradesh. *J. Orchid Soc. India*, **35**: 25-33.

Thakur, B. and Promila Pathak. 2020. *In vitro* propagation of *Herminium lanceum* (Thunb. ex Sw.) Vuijk (Orchidaceae), through asymbiotic seed germination: a therapeutically important and endangered orchid from NorthWestern Himalayas. *J. Orchid soc. India*, **34**: 61-67.

Thakur, B. and Promila Pathak. 2021. Application of organic additives for the enhancement of seed germination and seedling development in an endangered and medicinal orchid, *Rhynchostylis retusa* (L.) Blume through asymbiotic culture. *J. Orchid Soc. India*, **35**: 99-107.

Thant, M.T., B. Sritularak, N. Chatsumpun, W. Mekboonsonglarp, Y. Punpreuk, and K. Likhitwitayawuid. 2021. Three novel biphenanthrene derivatives and a new phenylpropanoid ester from *Aerides multiflora* and their á-glucosidase inhibitory activity. *Plants*, **10**(2): 385. https://doi.org/10.3390/plants10020385

Thapa, B., P. Sharma, S. Pradhan, and P. Pradhan. 2022. *Aerides multiflora* Roxb. an important ornamental and medicinal orchid. *J. Ayurv. Herbal Med.*, **8**(4): 236-40.

Thomas, T. D. and A. Michael. 2007. High-frequency plantlet regeneration and multiple shoot induction from cultured immature seeds of *Rhynchostylis retusa* Blume., an exquisite orchid. *Plant Biotechnol. Rep.*, **1**(4): 243-49.

Tikendra, L., T. Amom, and P. Nongdam. 2018. Effect of phytohormones on rapid *in vitro* propagation of *Dendrobium thyrsiflorum* Rchb.f.: An endangered medicinal orchid. *Pharmacogn. Mag.*, **78**: http://www.phcog.com.

Tikendra, L., N. Apana, A. M. Potshangbam, T. Amom, R. Choudhary, R. Sanayaima, A. Dey, and P. Nongdam. 2020. *Dendrobium* sp.: *In vitro* propagation of genetically stable plants and ethno-medicinal uses. *In: Orchids Phytochemistry, Biology and Horticulture: Fundamentals and Applications*. pp. 1-42. Springer, Switzerland.

Valencia-Glushchenko, N., C. G. Oña-Arias, M. Orellana, M. Ortega, A. Montero-Oleas, and M. de Lourdes Torres. 2024. *In vitro* asymbiotic seed germination and seedling development of four endangered Ecuadorian orchids: *Epidendrum Jamiesonis*, *Pleurothallis pulchella*, *Oncidium pentadactylon*, and *Elleanthus capitatus*. *PCTOC.*, **158**(3): 60.

Vasundhra. 2023. *Assessment of Encapsulating Technology and Conservation of Selected Commercially Important and Endangered Northwestern Himalayan Orchids: An In Vitro Study*. Ph.D. Thesis, Panjab University, Chandigarh, India.

Vasundhra, Promila Pathak, and Anuprabha. 2021. *In vitro* asymbiotic seed germination and regeneration competence of leaf explants in *Satyrium nepalense* D.Don, a medicinally important, and an endangered terrestrial orchid of Kasauli hills, Himachal Pradesh (Northwestern Himalayas). *J. Orchid Soc. India*, **35**: 73-82.

Verma, Shivani. 2016. *Influence of Different Growth Additives on In Vitro Asymbiotic Seed Germination, Micropropagation and Related Morphogenetic Stages in Some Medicinally Important Orchids.* Ph.D. Thesis, Panjab University, Chandigarh, India.

Vij, S. P. and K. S. Malhotra. 1988. Asymbiotic seed culture of *Acampe papillosum* Lindl. *In: National Seminar on Current*

Research Trends in Indian Orchids with a Special Reference to Tissue Culture Technology (ed. S. P. Vij and S. P. Khullar) pp. 14. The Orchid Society of India, Chandigarh, India.

Vij, S. P. and Promila Pathak. 1988. Green pod culture of *Pholidota articulata* Lindl. *In: Proc. National Seminar on Current Research Trends in Indian in Indian Orchids with a Special Reference to Tissue Culture Technology* (eds. S. P. Vij and S. P. Khullar) pp. 11. The Orchid Society of India, Chandigarh, India.

Vij, S. P., Promila Pathak, Mahant K. C. 1995. Green pod culture of a therapeutically important species *Dactylorhiza hatageria* D. Don Soo. *J Orchid Soc India*, **9**:7-12

WFO. 2023. World Flora Online. Published on the Internet; http://www.worldfloraonline.org.

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. *Sci. Hortic.*, **138**: 198-209.