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GREEN POD CULTURE OF AN ENDANGERED AND MEDICINALLY IMPORTANT ORCHID, *VANDA CRISTATA* WALL. EX LINDL. FROM HIMACHAL PRADESH

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Abstract

Himachal Pradesh, situated between the latitudes 30°22' to 33°12' N and longitudes 75°4' to 79°4' E, supports natural, unique, and socio-economically important orchids represented by 84 species in 43 genera. *Vanda cristata* commonly known as *Comb Vanda* and *Comb Trudelia*, is an epiphyte with high medicinal and horticultural values. It is used as tonic and expectorant and also to treat fever, cough, bronchitis, tonsillitis *etc*. It also has anticancerous and antibacterial properties. Because of the over-exploitation for the medicinal properties of this plant, natural populations are declining at a very rapid pace. Keeping this in mind, presently an attempt was made to culture immature seeds procured from green capsules (27 wap) on half and full strength MS (Murashige and Skoog, 1962) nutrient media augmented with different growth hormones such as auxins (NAA- 2.6 µM and 5.3 µM) and cytokinins (BAP- 2.2 µM and 4.4 µM) for its mass propagation. Cent per cent germination was observed in all the nutrient combinations of both half and full strength MS media. ½MS basal medium showed earliest seed germination (9.25±0.25 days), favoured formation of green and healthy protocorms. MS medium supplemented with NAA (5.3 µM) induced early rooting and complete seedling development was observed in 61.25±0.47 days of inoculation. In another experiment, 2-3 leaved shoot buds were subcultured on ½MS medium supplemented with auxins (NAA- 2.6 µM and 5.3 µM /or IAA- 2.8 µM and 5.7 µM) to assess the effect of these hormones on the rooting of *V. cristata*. ½MS medium augmented with IAA (5.7 µM) was observed as an optimal nutritional combination for early root formation (5.25±0.25 days). The protocol can be successfully utilized for rapid mass multiplication of *V. cristata* and aid in alleviating the collection pressures on its natural populations.

Introduction

THE INDIAN Himalayan Region (IHR) is covered by snow-clad peaks, glaciers of higher Himalayas, and dense forest cover of mid Himalayas (Devi *et al.,* 2018; Prakash and Pathak, 2020) and one of the orchid rich belts of India along with more than 900 species (Kumar *et al.,* 2018). Himachal Pradesh is situated between the latitudes 30°22' to 33°12' N and longitudes 75°4' to 79°4' E, covering area of 55,673 km^2. The climate varies from hot and sub humid to cold alpine, supports natural, unique, and socio-economically important orchids represented by 84 species in 43 genera (Singh *et al.,* 2019). Like other parts of IHR, orchids of Himachal Pradesh are well known for their delightful beauty and efficacy (Barman *et al.,* 2016). Attempts were also made to study the diversity and ecology of orchids of Himachal Pradesh (Devi *et al.,* 2018; Kumar *et al.,* 2017, 2018, 2019; Kumari and Pathak, 2020; Lal and Pathak, 2020; Pathak *et al.,* 2010; Prakash and Pathak, 2019; Prakash *et al.,* 2018; Sharma *et al.,* 2017; Singh *et al.,* 2019; Vij *et al.,* 2013).

Vanda R.Br. (Family- Orchidaceae; Subfamily- Epidendroideae; Tribe- Vandeae; Subtribe- Aeridinae) consists of more than 73 species (Chase *et al.,* 2015) of monopodial epiphytic orchids distributed in India, China, Sri Lanka, Philippines, and throughout South East Asia (De *et al.,* 2015). The name *Vanda* is originated from Sanskrit language, under the name *Rasna* in Ayurvedic formulations and representatives of this genus are used in the treatment of rheumatic pain, ear infection, and nervous system disorders (Hossain, 2011). *Vanda* species are also used in cosmeceuticals (Sharma and Pathak, 2020). *Vanda cristata* (Syn *Luisia striata* and *Trudelia cristata*) commonly known as *Comb Vanda*, *Comb Trudelia*, is found at altitudinal range of 1000-2000 m amsl in Himalayas. In India, it is distributed in Himachal Pradesh, Uttarakhand, Bihar, Madhya Pradesh, West Bengal, and NorthEast India. It has thick and stout stem, leaves are linear-oblong, fleshy, and unequally bilobed at the apex. Yellowish, with purplish blotches, the flowers are arranged in raceme, axillary inflorescence (Vij *et al.,* 2013). *V. cristata* is medicinally and horticulturally very important plant (Chand *et al.,* 2020). Paste prepared from whole plant is applied in cuts and wounds (Manandhar, 2002). Leaves are used to treat fever, cough, bronchitis, tonsillitis and also as tonic and expectorant (Medhi and Chakrabarti, 2009; Rao, 2004; Sharma *et al.,* 2017). Paste prepared from roots is applied to treat boils and dislocated bones. It also has anticancerous and antibacterial properties (Joshi *et al.,* 2020; Manandhar, 2002; Pant, 2013).

Because of the predictable medicinal properties of *V. cristata*, it is over-exploited from nature. Due to

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this, its natural populations are rapidly declining and it is included in Appendix-II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 2017). Some attempts were made to propagate and conserve a few medicinally as well as floriculturally important orchid species through asymbiotic seed culture (Anderson, 1990; Anuprabha and Pathak, 2012, 2019; Bhatti *et al.,* 2017; Bhowmik and Rahman, 2020a; Bustam *et al.,* 2016; Lekshmi and Decruse, 2018; Gurudeva, 2019; Kaur *et al.,* 2017; Pathak, 1989; Pathak *et al.,* 2001, 2016; Neupane *et al.,* 2020; Thakur and Pathak, 2020) and also by other explants such as leaf (Bhattacharjee and Islam, 2017; Chookoh *et al.,* 2019; Pathak *et al.,* 2017; Seeni and Latha, 2000; Sembi *et al.,* 2020), stem (Arora *et al.,* 2016; Bhattacharjee and Islam, 2017; Kaur, 2017), inflorescence (Liao *et al.,* 2011; Vasundhra *et al.,* 2019; Vij *et al.,* 1997), and pseudobulb (Anuprabha and Pathak, 2020; Anuprabha *et al.,* 2017; Bhowmik and Rahman, 2020b; Kaur and Bhutani, 2013; Vij and Pathak, 1989) *etc*.

Hence, the present study was carried out to standardize the protocol for efficient mass propagation and conservation of an endangered and medicinally important orchid, *Vanda cristata* using green capsules (pods) culture technique.

Material and Methods

Collection of Plant Material

Plants were collected from Kalapul (Distt. Kangra, co-ordinates range between 32°13' N to 76°19' E), Himachal Pradesh, India, during the month of June (2018) and planted in the Orchid Green House, Department of Botany, Panjab University, Chandigarh. Flowers were hand-pollinated in the last wk of February 2020 and green capsules (27 wap) were collected during the month of September 2020.

Seed Sterilization

In the present study, immature seeds procured from green capsules were used as explants. The green capsules were first scrubbed with Teepol (0.01%) for 5 min, washed thoroughly under running tap water for 20-25 min, dipped in 75% ethanol for 30 sec, flamed and then surface sterilized for 5 min with $HgCl_2$ (0.1%) solution, followed by 4-5 repeatedly washing with sterilized distilled water to remove all the traces of sterilizing agents. The sterilized capsules were then split open longitudinally with a sterilized blade to scoop out immature seeds, under aseptic conditions, in a laminar air flow cabinet.

Culture Media

The germination potential of immature seeds was tested on half strength (½MS) and full strength MS (Murashige and Skoog, 1962) media and its combinations with and without growth hormones such as auxins (NAA- 2.6 µM and 5.3 µM) (HiMedia Laboratories Pvt. Ltd.) and cytokinins (BAP- 2.2 µM and 4.4 µM) (HiMedia Laboratories Pvt. Ltd.). Sucrose (2% in ½MS and 3% in MS, Thermo Fisher Scientific, U.S.A.), was added as the carbohydrate source and the medium was gelled by using 0.8% agar (Thermo Fisher Scientific, USA), and pH of the medium was adjusted at 5.7±0.1 by using 0.1N NaOH or HCl. The medium was autoclaved at 121°C, 15 psi for 20 min.

Incubation Conditions

The sterilized immature seeds were inoculated on nutrient media. Cultures were maintained at 25±2°C temperature and exposed to 12 hr illumination of 3500 lux intensity.

Data Recording and Statistical Analysis

The experiment was repeated thrice and 4 replicates were used for each treatment. 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. Seed germination percentage was derived using the following formula:

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

Rooting

In vitro grown healthy shoots with 2-3 small leaves were tested for rooting on ½MS (Murashige and Skoog, 1962) medium and its combinations with and without growth hormones such as auxins (NAA- 2.6 µM and 5.3 µM; IAA- 2.8 µM and 5.7 µM). The effect of these hormones on the rooting of *V. cristata* shoot buds was assessed.

Hardening of Seedlings

Healthy seedlings with 3-4 well grown leaves and 2-3 roots were gradually hardened *in vitro*, by sequential elimination of growth additives, vitamins, sucrose, and minor salts from the nutrient matrix at 20 days interval. The hardened seedlings were washed thoroughly with lukewarm water to remove agar and potted in pots, using charcoal, moss and brick-pieces (1:1:1) as the potting media and their survival rate was observed.

Results and Discussion

The immature seeds procured from green capsules (27 wap) were found to be monoembryonate and these were inoculated on ½MS and MS media using green pod culture technique with and without growth hormones. Plant growth regulators are considered to play significant role in all aspects of plant growth and development. According to Arditti (1967), the nutritional requirements of different orchids depend upon their inherent genetic makeup. The seeds showed cent per cent germination in all the tested combinations. The first sign of germination was swelling of embryos, followed by apical rupturing of seed coats, protocorm formation, and subsequent development into seedlings. The time taken for onset of germination, spherule formation, protocorms development and differentiation and seedling formation varied depending upon the PGRs used in the media (Table 1-2; Fig. 1A-I). Asymbiotic seed germination has emerged as a significant tool to mass propagate a large number of orchid species and hybrids (Arditti *et al.,* 1982) and it has been positively tested in some orchid species (Anuprabha and Pathak, 2012; Bhatti

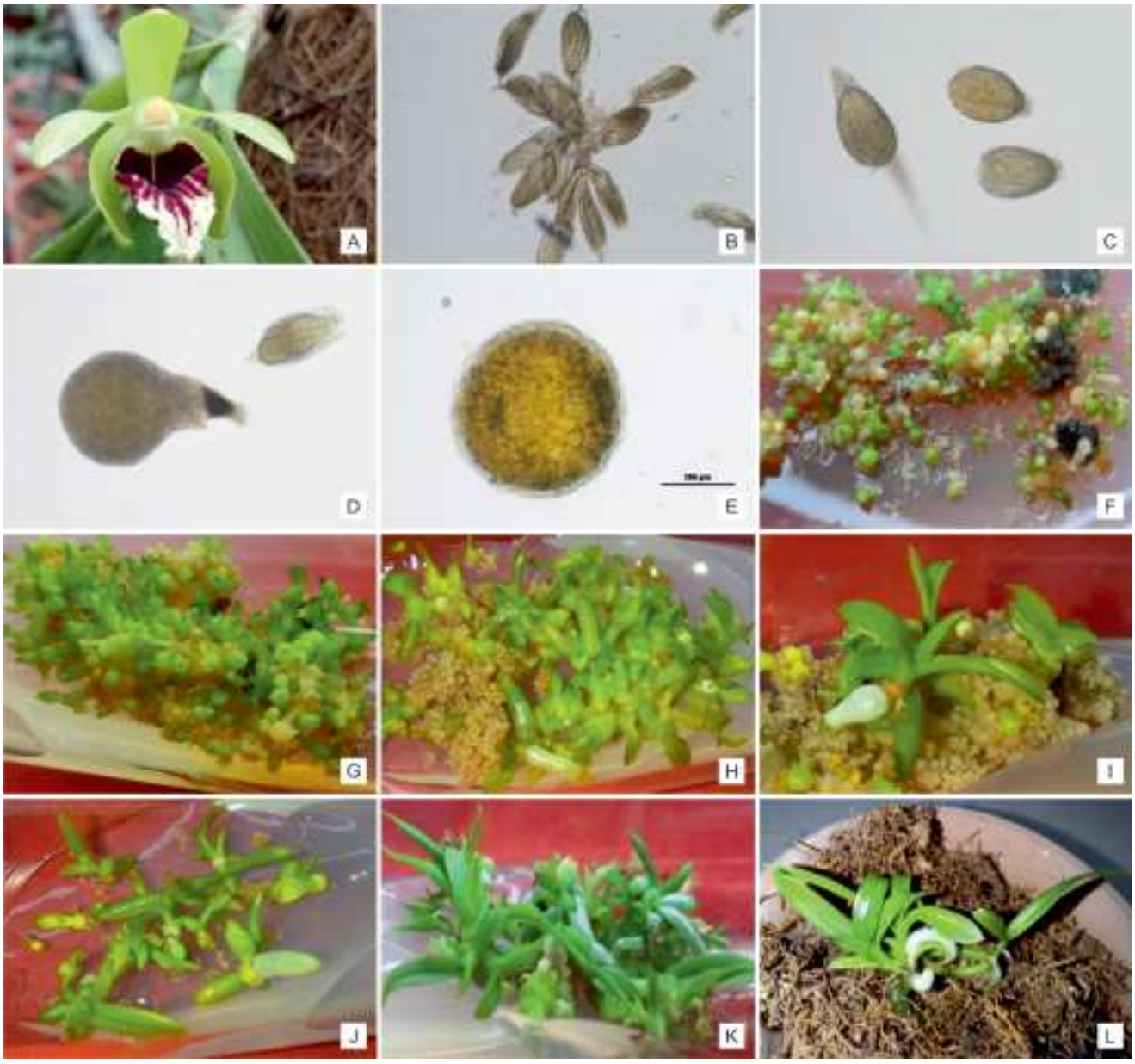

Fig. 1. A-L. Green pod culture in *Vanda cristata*: A, A single flower; B, Immature seeds at the time of inoculation; C, Swelling and apical rupturing; D, Spherule formation; E, Chlorophyll synthesis in spherule [½MS (Control)]; F-G, Protocorm development and multiplication [½MS (Control)]; H, Leaf and root differentiation [MS+ NAA (5.3 µM)]; I, Seedling development [MS+ NAA (5.3 µM)]; J-K, Root induction in *in vitro* grown shoot buds [½MS+IAA (5.7 µM)]; L, Hardened seedlings transferred to a pot.

et al., 2017; Bhowmik and Rahman, 2020a; Bustam *et al.,* 2016; De *et al.,* 2006; Lekshmi and Decruse, 2018; Giri and Tamta, 2012; Gurudeva, 2019; Madhavi and Shankar, 2019; Mahant, 1991; Mohanty and Salam, 2017; Pathak, 1989; Pathak *et al.,* 2001, 2016; Neupane *et al.,* 2020; Thakur and Pathak, 2020; Vij and Pathak, 1988; Vij *et al.,* 1995).

The ½MS basal medium showed early seed germination (9.25±0.25 days). During germination, the embryo swelled and emerged out of the seed coats through apical rupturing as spherules (14.75±0.25 days) followed by green and healthy protocorm formation (20.75±0.47 days). Subsequently, the protocorms differentiated into leaf and root primordia (25.50±0.28 and 53.50±0.64 days, respectively) and complete healthy seedlings were obtained in 71.25±0.75 days. Augmentation of auxin *i.e.* NAA (2.6 µM) in ½MS medium though delayed the onset of germination (13.25±0.25 days), spherule formation (17.25±0.25 days), and leaf primordia development (27.50±0.28 days), helped in early root formation (51.25±0.25 days) and seedling development (66.75±0.47 days). While medium supplemented with NAA (5.3 µM) delayed all the morphogenetic changes of seed germination, incorporation of cytokinin *i.e.* BAP (2.2 and 4.4 µM) in ½MS medium induced protocorm multiplication but delayed the onset of seed germination (12.25±0.47 and 12.00±0.40 days, respectively) and inhibited root development. Jualang *et al.* (2014) found NAA (0.1 mgl^{-1}) as the best PGR for enhancing seed germination in *Vanda dearie*. Koirala *et al.,* (2013) also observed that NAA (0.5 mgl^{-1}) supports early seed germination and protocorm formation in *Coelogyne fuscescens*. Manokari *et al.* (2021) observed highest seed germination in *Vanda tessellata* using BAP (1.5 mgl^{-1}). Basker and Narmatha (2010) proved that BAP (2.0 mgl^{-1}) was beneficial for production of multiple protocorms but failed to produce roots in all the tested concentrations. Similarly, Manners *et al.* (2011) observed highest percentage of seed germination in the presence of BAP (5.0 µM), in the medium.

In MS basal medium, seeds germinated within 10.75±0.47 days of inoculation and further development of complete well rooted seedlings was observed within 73.25±0.75 days. When MS medium amalgamated with NAA (2.6 µM) slightly delayed complete seedling formation. MS medium containing NAA (5.3 µM) though proved ineffective during onset of germination, protocorm formation, and differentiation, proved beneficiary for early root formation (49.25±0.47 days), and complete seedling formation (61.25±0.47 days). While BAP (2.2 µM and 4.4 µM) in MS medium though delayed the onset of seed germination and protocorm formation, inhibited root formation, favoured formation of healthy chlorophyllous protocorms. Similarly, Nanekar *et al.* (2014) reported that NAA (0.5 mgl^{-1}) was suitable for higher seed germination in *Eulophia nuda*. In *Malaxis acuminata,* NAA (4 µM) proved the best for early immature seed germination (Arenmongla and Deb, 2012). According to Pant *et al.,* (2011), BAP (0.5 mgl^{-1}) was found to be the most suitable for immature seed germination and protocorm development in *Phaius tankervilleae*. Nagarju *et al.* (2003) reported that proliferated protocorms were developed in *Cattleya* and *Cymbidium* when supplemented with BAP (0.5 mgl^{-1}). Similar results have been obtained by De Pauw *et al.* (1995) on *Cypripedium candidum* seed germination where BAP (0.8 mgl^{-1}) enhanced faster germination and induced

Table 1. Effect of different growth hormones on *in vitro* seed germination and seedling development in *Vanda cristata* on ½MS medium.

Growth hormones	Germination frequency	Onset of germination (in days)	Time taken in days for development of					Remarks
			Spherule formation	Protocorm formation	Emergence of 1st leaf primordium	Emergence of 1st root primordium	Seedlings	
-	100%	9.25±0.25^{a}	14.75±0.25^{a}	20.75±0.47^{a}	25.50±0.28^{a}	53.50±0.64^{b}	71.25±0.75^{b}	Green and healthy protocorms
NAA (2.6 µM)	100%	13.25±0.25^{bc}	17.25±0.25^{b}	23.00±0.70^{ab}	27.50±0.28^{bc}	51.25±0.25^{a}	66.75±0.47^{a}	Early seedling development
NAA (5.3 µM)	100%	14.00±0.40^{c}	17.00±0.40^{b}	24.25±0.47^{b}	28.00±0.40^{c}	60.00±0.40^{c}	80.25±0.69^{c}	Delayed seedling development
BAP (2.2 µM)	100%	12.25±0.47^{b}	16.25±0.62^{ab}	23.25±0.47^{b}	26.00±0.40^{ab}	-	-	Protocorm multiplication
BAP (4.4 µM)	100%	12.00±0.40^{b}	16.25±0.64^{ab}	22.75±0.75^{ab}	26.50±0.28^{b}	-	-	Protocorm multiplication

Entries in column number 3 to 8 are Mean±S.E.; same alphabetical letter in the superscript denotes that the corresponding means are in the same group suing Tukey test at 5%.

Table 2. Effect of different growth hormones on *in vitro* seed germination and seedling development in *Vanda cristata* on MS medium.

Growth hormones	Germination frequency	Onset of germination (in days)	Time taken in days for development of					Remarks
			Spherule formation	Protocorm formation	Emergence of 1st leaf primordium	Emergence of 1st root primordium	Seedlings	
-	100%	10.75±0.47^{a}	14.75±0.47^{a}	22.00±0.40^{a}	28.00±0.40^{ab}	54.00±0.40^{b}	73.25±0.75^{b}	Well rooted seedlings
NAA (2.6 µM)	100%	11.25±0.47^{a}	16.25±0.85^{ab}	23.25±0.47^{ab}	30.00±0.40^{b}	54.50±2.32^{b}	77.50±0.64^{c}	Well rooted seedlings
NAA (5.3 µM)	100%	12.50±0.64^{ab}	15.50±0.64^{ab}	23.00±0.40^{ab}	27.25±0.47^{a}	49.25±0.47^{a}	61.25±0.47^{a}	Early seedling development
BAP (2.2 µM)	100%	13.50±0.28^{b}	17.75±0.25^{b}	25.00±0.70^{b}	29.50±0.64^{b}	-	-	Healthy protocorms
BAP (4.4 µM)	100%	12.00±0.40^{ab}	16.00±0.40^{ab}	23.00±0.70^{ab}	26.50±0.28^{a}	-	-	Green and healthy protocorms

Entries in column number 3 to 8 are Mean±S.E.; same alphabetical letter in the superscript denotes that the corresponding means are in the same group suing Tukey test at 5%.

protocorm multiplication. BAP (1.0 mgl^{-1}) showed its benign effect during seed germination in *Dendrobium aphyllum* (Hossain *et al.,* 2013).

The half strength MS medium supplemented with four different combinations of auxins (NAA, IAA) was used for induction of roots (Table 3). The efficiency of the rooting medium was observed the best (5.25±0.25 days) for root formation when it was augmented with 5.7 µM IAA (Fig. 1J-K). Induction of roots is an inherent nature of plants which is controlled by endogenous level of hormones. Exogenous supply of root inducing hormones like auxins enhanced the process. The results of the present findings suggested that the combined effects of nutritional stress with IAA has been reported to be the most appropriate in inducing roots in some epiphytic orchids (Bhadra *et al.,* 2002, 2004; Bhattacharjee and Islam, 2014; Bhowmik and Rahman, 2020a; Dutta *et al.,* 2011; Hoque *et al.,* 1994; Hossain, 2013; Hossain *et al.,* 2009, 2010; Nayak *et al.,* 1997).

In the present investigation, a successful attempt was made to study the *in vitro* seed germination of *V. cristata* and their subsequent development into seedlings. From the above data, full strength MS medium with NAA (5.3 µM) was found as the best nutrient medium for early seedling development. Cent per cent seed germination was, however, observed on both the nutrient media. The study is in agreement with earlier studies made on some orchid species including *Cleisostoma racemifefum* (Deb and Temjensangb, 2007), *Cymbidium aloifolium* (Hossain *et al.,* 2009; Pradhan *et al.,* 2013), *Cymbidium mastersii* (Mohanty *et al.,* 2012), *Gastrochilus calceolaris* (Pathak *et al.,* 2011), *Malaxis khasiana* (Deb and Temjensangba, 2006), *Phaius tankervilleae* (Pant *et al.,* 2011; Thokchom *et al.,* 2017), *Spathoglottis plicata* (Reddy *et al.,* 1992), *Vanda coerulea* (Hrahsel and Thangjam, 2015; Manners *et al.,* 2011), and *Vanda tessellata* (Prakash *et al.,* 2013). On the other hand, a few scientists have suggested ½MS medium to be the best for seed germination and differentiation in *Dendrobium candidum* (Liu and Zhang, 1998), *Vanda dearie* (Jualang *et al.,* 2014), *Vanda tessellata* (Madhavi and Shankar, 2019).

In vitro raised seedlings were gradually hardened and potted in pots, using charcoal, moss and brick-pieces

Table 3. Effect of auxins on rooting and seedling development in *Vanda cristata* on ½MS medium.

Growth hormones	Time taken in days for development of		Remarks
	Root primordium	Seedlings	
-	11.50±0.95^{b}	28.75±0.85^{b}	Healthy seedling formation
NAA (2.6 µM)	17.75±0.62^{c}	36.00±0.57^{c}	Healthy seedling formation
NAA (5.3 µM)	24.25±0.47^{d}	42.75±0.25^{d}	Delayed rooting
IAA (2.8 µM)	15.50±0.50^{c}	33.75±0.75^{b}	Healthy seedling formation
IAA (5.7 µM)	5.25±0.25^{a}	19.75±0.75^{a}	Early rooting

Entries in column number 2 and 3 are Mean±S.E.; same alphabetical letter in the superscript denotes that the corresponding means are in the same group suing Tukey test at 5%.

(1:1:1) as the potting media and 70% survival rate was observed. The older the seedlings, the broader was the tolerance range for various atmospheric conditions.

Conclusion

Green Pod Culture technique is an outstanding technique to mass propagate medicinal and threatened orchid species. During the present study, the growth hormones showed a stimulating effect on seed germination, growth and multiplication of protocorms and their differentiation into seedlings. Based on the above results, ½MS medium is recommended for the early asymbiotic seed germination and healthy protocorm formation in *Vanda cristata* whereas MS medium supplemented with NAA (5.3 µM) was proved as the best for early and healthy seedling development. IAA supplemented with (5.7 µM) in ½MS basal medium enhanced early and healthy rooting.The suggested protocol can be utilized for rapid mass multiplication of the presently investigated species, *V. cristata* and will aid in alleviating the collection pressures on its natural populations.

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