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Asymbiotic *In vitro* Seed Germination and Regeneration of *Vanda coerulea* Giff. Ex. Lindl., an Endangered Orchid from Northeast India

Research Article

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Abstract

Blue Vanda (*Vanda coerulea* Giff. ex. Lindl.) is one of the most beautiful orchids found in the northeastern region of India. Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) listed this species in the appendix 1 as a vulnerable one. In the present study asymbiotic *in vitro* seed germination and regeneration of *V. coerulea* via protocorm formation was achieved in MS basal medium. Immature seeds cultured on full strength MS basal medium showed higher rate of germination with subsequent formation of 'protocorm like bodies' (PLB) in comparison to half strength MS basal medium. Multiplication of the protocorms and differentiation into shoots was attained in MS basal medium supplemented with combination of benzylamino purine (BA) and kinetin (Kn). Highest number of leaf emergence was observed in MS basal medium supplemented with 22.80 µM indole acetic acid (IAA). Supplementation of basal medium with banana extract (75 mg L^{-1}) resulted in highest number of root induction. The standardized protocol for efficient *in vitro* germination of immature *V. coerulea* seeds and their subsequent regeneration can be applied for future mass propagation and *ex situ* conservation of this endangered orchid.

Keywords: *Vanda coerulea; In vitro* seed germination; Protocorm-like bodies; Regeneration

Introduction

Orchids are famous for their beautiful range of flowers, not only for their aesthetic values but also for pharmaceutical importance. Orchid seeds are dispersed by wind far and wide and if fall into new environmental condition, they either die or adapt to the new environment [1]. Some members of orchids have also been used for their therapeutic value owing to their phytochemical constituents such as alkaloids, flavanoids, terpenes and glycosides [2]. The orchid seeds are minute and produced in million per capsule but lacks metabolic machinery and functional endosperm and thus only 0.2 - 0.3% of the seeds germinate in nature [3].

Vanda coerulea Giff. ex Lindl., commonly known as Blue Vanda or Autumn lady's tresses orchid, is a remarkably beautiful plant having large pale to deep liliac-blue flowers (Figure 1A). It produces an erect stem 2 to 3 feet high with the usual distichous loriform channeled coriaceous leaves, which are unequally truncate with a concave notch and acute lateral lobes. The erect scapes are longer than the leaves and bear dense racemes of 10-15 flowers. The flowers are produced during September to November, last for 2 to 3 months and shows change in coloration from light blue to darker shades of blue. This species is distributed in northeast India, China, Burma and Thailand [4]. Due to its high commercial value in the local and international flower market it is harvested indiscriminately. The reckless collection and ever increasing habitat destruction and global warming have posed direct threats to its existence. *V. coerulea* has been listed under Appendix II (112/01/05) of CITES [5] and categorized as vulnerable [6]. It has been listed as an endangered species of Red Data Book on

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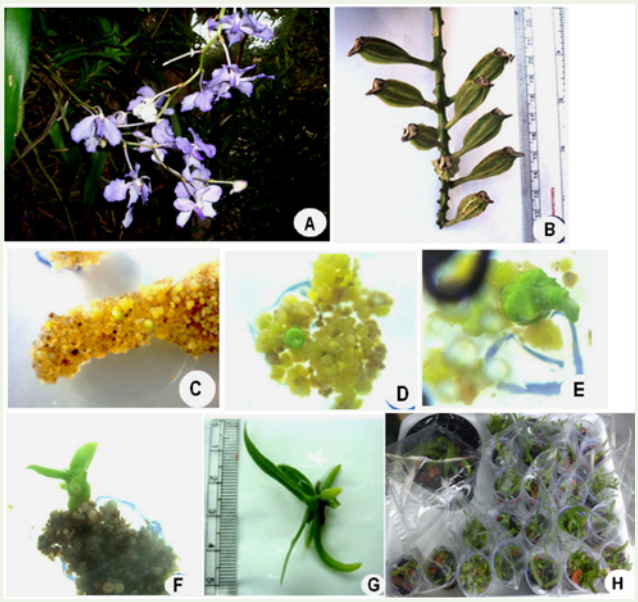

Figure 1: *In vitro* seed germination and regeneration of *Vanda coerulea* Giff. ex. Lindl. - **A)** *V. coerulea* in natural habitat. **B)** Immature capsule. **C)** *In vitro* germination of seeds in full strength MS basal media. **D)** Protocorm-like bodies (PLBs) formation in full strength MS basal medium. **E)** Shoot induction in full strength MS basal medium. **F)** Induction of leaf formation in full MS basal medium supplemented with combination of 4.44 µM BA + 4.70 µM Kn. **G)** Regenerated plantlet with fully formed leaves in full MS basal medium supplemented with 22.8 µM IAA. **H)** Regenerated plants ready for acclimatization.

Indian Orchidaceae-1 [7] and threatened by International Union for Conservation of Nature and Natural Resource [8]. There are numbers of selected clones of *V. coerulea* which are urgently needed to conserve for future breeding program [9].

Plant tissue culture technology helps to propagate plants of economic importance such as orchids and other ornamental plants in large numbers through meristem culture or by other *in vitro* methods. The earliest report of orchid tissue culture dates back to 1949 when it was demonstrated that *Phalaenopsis* plantlets could develop from the buds of inflorescence stalks [10]. However the credit of achieving mass clonal propagation of orchids goes to Morel who in 1960 [11] was successful in culturing *Cymbidium* shoot apices on Knudson C agar medium. Further successes in other species through shoot tip culture are reported in *Aranda* [12], *Aranthera* [13], *Rhynchostylis* [14], *Dendrobium* [15,16] and *Vanda* [12]. The *in vitro* technique of asymbiotic seed germination could be useful for conservation and reintroduction of *V. coerulea* like other orchids [17,18]. Knudson in 1946 [19] revolutionized the technique of *in vitro* asymbiotic seed germination for orchid cultivation. Various explants have been used for the *in vitro* regeneration of plantlets [20]. However, shoot tip based micro propagation system, which is the main mode of propagation for sympodial orchids, is not optimal for this monopodial species because only a few axillary buds develops at the lower half of the plant and the continual growth from the apical meristem inhibits development of axillary buds into offshoots, and hence resulting in low rate of vegetative multiplication under natural conditions [22]. The removal of meristem tips also greatly endangers the survival of the mother plant [20,21]. Thus the present study of *in vitro* regeneration of *V. coerulea* found in Mizoram, northeast India was undertaken using asymbiotic seed germination techniques.

Materials and Methods

Plant material

Immature green pods of *V. coerulea* (Figure 1B) were collected from Mission Veng, Aizawl, India during February and kept at 4 °C until use.

In vitro germination

The immature green pods were removed and washed thoroughly using few drops of detergent (labolene) in running tap water for 10-15 min. The pods were surface sterilized by immersion in 1% Bavistin (Carbendazim 50% w/w) for 10-15 min and rinsed with running tap water for 15 min. They were then brought to the Laminar Air Flow Chamber and treated with 0.1% Mercuric chloride for 15 min, 70% ethanol (v/v) for 10 minutes and rinsed thoroughly with sterile distilled water 4-5 times. They were then dried with a sterile blotting paper and cut longitudinally into two halves using a sterile blade. The immature seeds together with cotton fibres in between were scooped out into sterile petriplates. After careful separation of the immature seeds from the fibres with the help of a pair of sterile forceps, the immature seeds were transferred to petriplates containing variable strength of MS basal medium [23]. All the cultures were maintained at 25±1 °C continuous dark and 60-70% relative humidity. Observations were recorded every week for the production of protocorm-like bodies (PLBs) as a successful sign of *in vitro* germination.

In vitro regeneration, rooting and acclimatization

Protocorms which have developed from the immature seeds cultured on half and full strength MS medium were collected and then transferred to fresh basal MS medium supplemented with various concentrations of plant growth regulators (PGRs) viz., 6-Benzylamino purine (BA) and Kinetin (Kn) either singly or in combinations. The various concentrations of BA and Kn used are BA (4.44 µM), BAP (8.88 µM), Kn (4.70 µM), Kn (9.40 µM), BA (4.44 µM) + Kn (4.70 µM), BA (4.44 µM) + Kn (9.40 µM) and BA (8.88 µM) + Kn (4.70 µM). Similarly, PLBs were also cultured on half and full strength basal MS media without PGRs as control. The PLBs which were cultured on half MS and full strength MS basal media showing multiplication of protocorms and formation of shoots were transferred into different rooting media containing MS basal medium supplemented with indole acetic acid (IAA) and banana extract (75 mg L^{-1}). The concentration of IAA added in the MS basal medium varies from 5.70 µM to 28.5 µM. The pH of the medium was adjusted to 5.8 prior to autoclaving at 15 psi and 121 °C for 20 min and was maintained at 25±2 °C under a 16 h photoperiod with a photosynthetic photon flux density of 35 µmol/m^2/s provided by cool white fluorescent tubes (Philips, India). They were sub-cultured every 2 weeks into a fresh media containing the same composition. For each treatment 3 replicates were taken. The individual and combined effects of these PGRs were analyzed through parameters such as the multiplication of protocorms and formation of shoots and roots. All the chemicals

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used in this study were of HiMedia, Mumbai, India. The regenerated plantlets with well-developed roots were removed from the culture medium, washed gently under running tap water and transferred to plastic pots containing charcoal chips, brick pieces and sphagnum mosses. The pots containing the plantlets were then covered with transparent polyethylene bags to maintain adequate moisture for a week and transferred to the greenhouse (temperature: 25± 2 °C, light intensity: 4000 lux; photoperiod: 14-h; humidity: 70%). After 4 weeks, the plastic covering was removed and the plantlets were maintained in the greenhouse in plastic pots containing normal garden soil until they were transplanted to the nursery.

Statistical analysis

All the experiments had three replicates per treatment and were repeated at least twice. The differences in means were contrasted using Duncan's new multiple range test following ANOVA. All statistical analysis was carried out using SPSS statistical software package version 16.0.

Results

In vitro seed germination and PLB formation

In vitro seed germination and their subsequent regeneration of *V. coerulea* cultured on half and full strength MS media showed variable response. Seeds cultured on half strength MS basal medium showed first response i.e. swelling of immature seeds by the 4th week whereas in the full strength MS medium the initial response was observed by the 7th week, which was followed by pigment synthesis (Figure 1C). The colour change observed in the seeds were observed as follows: creamy yellow within 9th week in the half strength MS medium whereas in full strength MS medium it was observed within 6th week then greenish yellow within the 12th week in the half strength MS medium and 7th week in the full strength MS medium. The formation of protocorm-like bodies (PLBs) was observed in the 14th week in half strength MS medium whereas it was observed in the 12th week in full strength MS medium. PLBs are outgrowths that arise from seeds which act as transitory bodies leading to *in vitro* regeneration of orchids (Figure 1D). Thus, the immature seeds cultured in full MS medium showed higher response than those cultured in half strength MS medium. This showed that the seedling development of *V. coerulea* was better on full MS medium.

The *in vitro* germinated seeds of *V. coerulea* on subsequent transfer to half and full strength MS medium supplemented with various concentrations of BA and Kn either singly or in combinations showed variable response. The half MS derived PLBs showed their first response i.e. multiplication of PLBs within the 2nd week in media containing 4.44 µM BA, 4.70 µM Kn, 9.40 µM Kn, 4.44 µM BA + 4.70 µM Kn, 4.44 µM BA + 9.40 µM Kn and 8.88 µM BA + 4.70 µM Kn. In the control (full strength MS without PGRs) the same observation was recorded by the 4th week in control. On the other hand the full strength MS media derived PLBs showed the first response in the 2nd week in all the media composition.

Shoot induction and leaf formation

The half MS derived PLBs showed shoot induction in 3 weeks in the media containing 4.44 µM BA, 4.44 µM BAP + 4.70 µM Kn and 8.88 µM BA + 4.70 µM Kn whereas it took 4 weeks in media containing 9.4 µM Kn, 4.44 µM BA + 9.40 µM Kn. Shoot induction was not observed in the control media as well as those supplemented with 8.88 µM BA and 4.70 µM Kn. While the full strength MS derived PLBs showed shoot induction by 3rd week in control as well in the media supplemented with 4.44 µM BA, 4.70 µM Kn, 9.40 µM Kn, 4.44 µM BA + 4.70 µM Kn, 4.44 µM BA + 9.40 µM Kn, 8.88 µM BA + 4.70 µM Kn. But in the 9.40 µM Kn and 4.44 µM BA + 9.40 µM Kn supplemented MS media it was observed by the 4th week. No shoot induction was observed in the media supplemented with 8.88 µM BA only. Leaf formation for the half MS medium derived PLBs was observed in 4 weeks only in one media combination i.e. MS media containing combination of 8.88 µM BA + 4.7 µM Kn. Whereas the leaf formation for the full strength MS medium was observed in 4 weeks in the control as well as in the media supplemented with 4.44 µM BA + 9.40 µM Kn and 3 weeks in the media containing 4.44 µM BA + 4.70 µM Kn. For the development and multiplication of leaves, the shoots were sub-cultured in a fresh media containing the same composition. Details of the shoot induction and leaf formation are provided in Table 1 and Figure 1E, 1F, 1G & 1H.

Leaf multiplication, rooting and hardening of regenerated plantlets

The regenerated shoots which resulted in the formation of leaves were sub-cultured to different rooting media containing different concentrations of Indole Acetic Acid (IAA) and banana extract (75 mg L^{-1}). After 4 weeks of culture the multiplication of leaves and induction of roots started (Table 1). The total number of leaf formation observed after 8 weeks of culture was found to be variable in the different media compositions. In the half strength MS media

Table 1: Effect of half and full strength MS media supplemented with indole acetic acid (IAA) and banana extract on leaf and root multiplication *V. coerulea*.

Sl. No.	IAA (µM)	Banana extract (mg L^{-1})	No. of leaves		No. of roots	
			Half strength	Full strength	Half strength	Full strength
1.	0.00	0.00	2.66 ± 0.33a	3.33 ± 0.33a	4.33 ± 0.33a	5.67 ± 0.67a
2.	0.00	75.00	3.33 ± 0.33ac	6.67 ± 0.66bc	12.33 ± 0.33bc	14.33 ± 0.33bc
3.	17.10	75.00	3.66 ± 1.52ad	4.33 ± 0.33ac	3.66 ± 0.33a	5.00 ± 0.57a
4.	5.70	0.00	4.67 ± 0.88ad	5.00 ± 0.57bde	3.67 ± 0.33a	4.66 ± 0.33a
5.	11.40	0.00	5.00 ± 0.57bcd	6.33 ± 0.33b	8.00 ± 0.57b	10.67 ± 0.67bde
6.	17.10	0.00	5.23 ± 0.55bcd	3.33 ± 0.33a	7.33 ± 0.88be	11.00 ± 0.57bd
7.	22.80	0.00	5.33 ± 0.88bcd	7.33 ± 0.33bf	9.33 ± 0.33bf	13.00 ± 0.58bfg
8.	28.50	0.00	4.66 ± 0.67acd	5.67 ± 0.33b	6.66 ± 1.20bg	8.66 ± 0.33bdfh

Mean (±) followed by the same letter(s) in each column of the same parameter were not significantly different at $P < 0.05$ using Duncan's new multiple range test.

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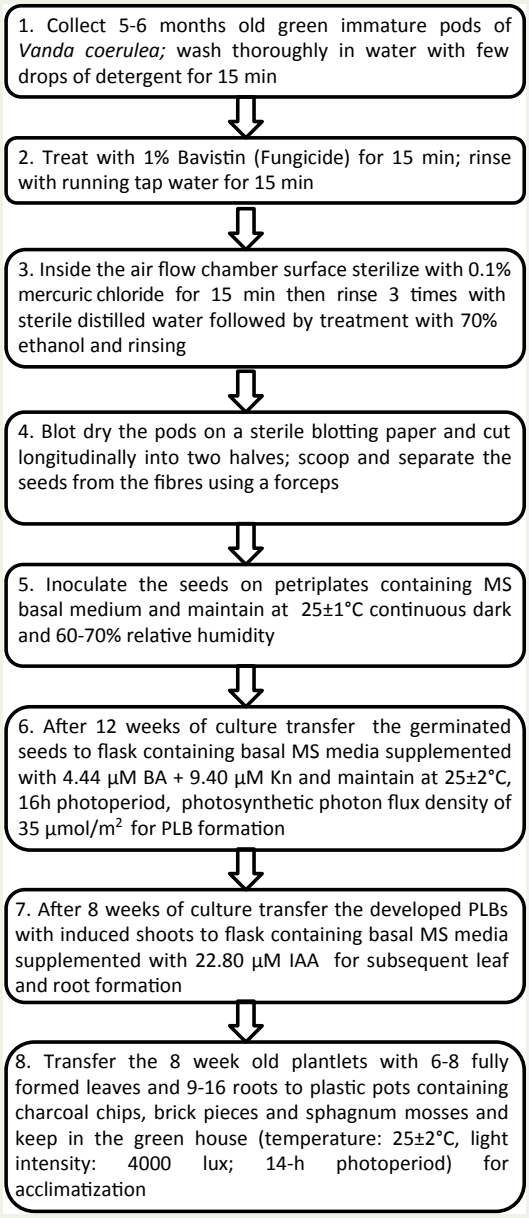

Figure 2: Schematic flow chart describing the protocol for *in vitro* seed germination and regeneration of *Vanda coerulea* Giff. ex. Lindl.

the mean number of leaves ranged from 2.66 (control) to 5.33 (22.8 µM IAA) while in the full strength MS media, it ranged from 3.33 (control) to 7.33 (22.8 µM IAA). The supplementation of banana extracts to MS media containing IAA did not show any incremental effect to the multiplication of leaves as evident by the lesser number of leaves recorded after 8 weeks of culture. The mean number of root formation observed after 8 weeks of culture in half and full strength MS basal media containing banana extract (75 mg L^{-1}) was 12.33 and 14.33 respectively (Table 1).

The fully rooted plantlets with at least 3 roots were taken out from the culture tubes, washed thoroughly to remove any remains of medium, and planted in small plastic pots containing charcoal chips, brick pieces and sphagnum mosses. The pots containing the plantlets were then covered with transparent polyethylene bags to maintain adequate moisture for a week and transferred to the greenhouse (temperature: 25± 2 °C, light intensity: 4000 lux; photoperiod: 14-h; humidity: 70%). After 4 weeks, the plastic covering was removed and the plantlets were maintained in the greenhouse in plastic pots containing normal garden soil until they were transplanted to the nursery. About 80% of the hardened plants survived in the nursery. The overall protocol standardized for the asymbiotic *in vitro* seed germination and its subsequent regeneration of *V. coerulea* in the present study are described in Figure 2.

Discussion

The present investigation indicated that *in vitro* germination of *V. coerulea* and their subsequent regeneration can be achieved successfully with minimum use of plant growth regulators. PLB formation was more suitable with full strength MS basal media without growth regulators. However, the multiplication of the PLBs and differentiation into shoots and leaves was favourable in basal MS supplemented with combination of BA and Kn in comparison to either BA or Kn alone. Highest number of leaf multiplication was observed in MS basal media supplemented with 22.80 µM IAA while supplementation with banana extract (75 mg L^{-1}) showed the highest number of root induction. The present standardized protocol for efficient *in vitro* germination and subsequent regeneration of immature *V. coerulea* seeds can be applied for future mass multiplication and conservation of this endangered orchid.

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