Propagation of *Vanda coerulea* Via *In Vitro* Asymbiotic Seed Germination

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ABSTRACT

An efficient method of propagation for *Vanda coerulea,* a rare and endangered species, was developed via *in vitro* asymbiotic germination of seeds. Seeds excised from 10 to 11-month old artificially pollinated capsules were cultured aseptically on different media. A maximum seed germination of 71.8% was recorded on Murashige and Skoog (MS) medium devoid of plant growth regulators. However, incorporation of either 5 µM 6-benzyl aminopurine (BAP) or 5 µM indole 3-acetic acid (IAA) separately in the MS medium further enhanced seed germination to 94.4% and 92.6%, respectively. A maximum shoot number of 13.2 with an average of 5.1 roots was obtained on MS medium supplemented with a combination of 5 µM BAP and 15 µM IAA. Well developed seedlings of *V. coerulea* were hardened in a potting medium comprised of charcoal, brick pieces and decaying litter in a 1:1:1 ratio, with a top layer of moss, wherein 91.2% survivability was obtained.

INTRODUCTION

Among horticultural and floral crops, orchids are outstanding in many ways as they have diverse shapes, forms and colors. Orchids are marketed as plants and as cut flowers and their production has increased tremendously in recent years (Tokuhara and Mii, 1993; Chang and Chang, 2000). However, orchids are quickly disappearing from their natural habitats due to deforestation, urbanization, utilization of land for agriculture and over-exploitation for commercial purposes. The minute orchid seeds are very difficult to germinate as they possess no endosperm and the embryo is usually immature. Because of their particular fungal requirement, less than 5% of orchid seeds germinate in nature (Rao, 1977). To save the diverse orchid species from extinction, *in vitro* culture techniques have been utilized to propagate plants (Hey and Hey, 1966).

Among the monopodial ornamental orchids, Vanda is very important from a commercial standpoint. *Vanda coerulea* Griff. ex Lindl. (Fig. 1a), popularly known as the 'Blue Vanda of Asia', is the progenitor of a vast variety of floriculturally significant hybrids due to its clear blue flowers. The species is also important ethnobotanically. The juice from its leaves is used to cure diarrhea, dysentery, and dermal disorders (Nadkarni, 1954). It is a perennial epiphyte growing at elevations of 1000-1500 m in the Khasi and Jaintia Hills of Meghalaya, India, and in the northern ranges of Thailand and Burma. Acclaimed as the 'Queen of the Vandas', it has been bred for such qualities as flower size, floriferousness, vigor and cold tolerance. Habitat destruction and overexploitation are the two important factors threatening its survivability in India (Pradhan,

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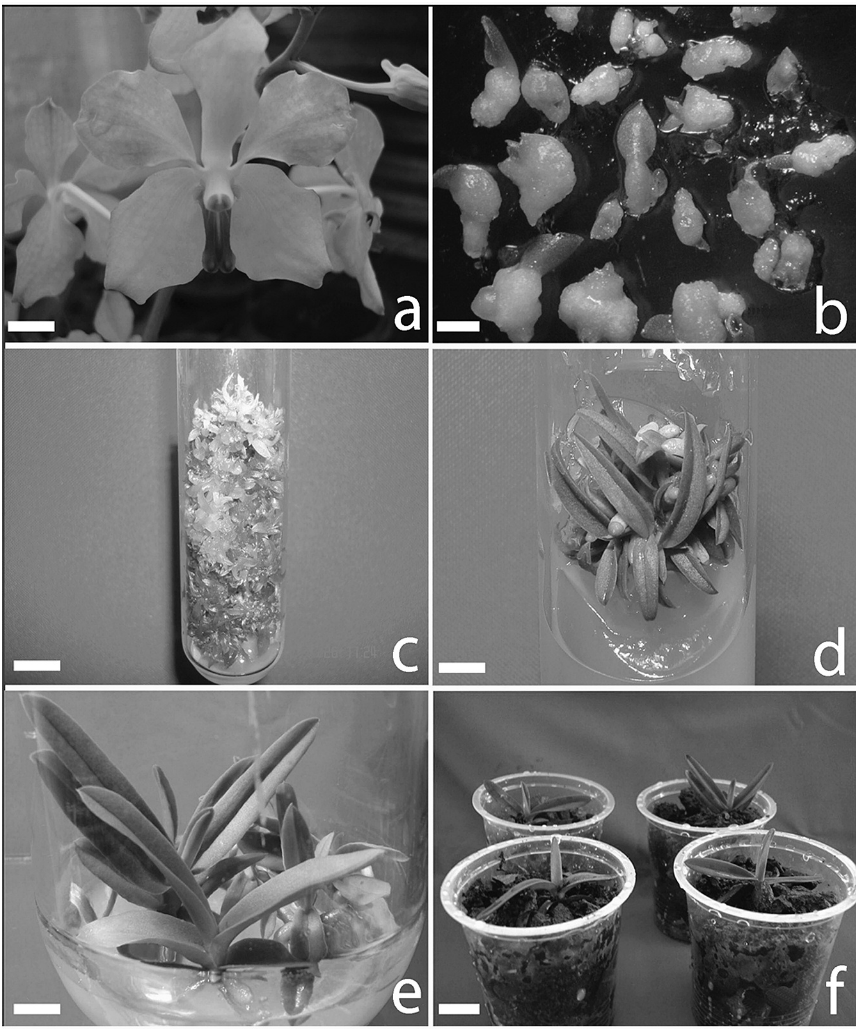
1985). It is listed in Appendix I of the "Committee for international trade in endangered species of wild fauna and flora" (Seeni and Latha, 2000), and included in the "Threatened plants list of India" published by the International Union for Conservation of Nature and Natural Resources (Devi et al., 1998;

Figure 1. Asymbiotic seed germination and propagation of *Vanda coerulea.* a. *Vanda coerulea* blooming (bar = 2 cm). b. Swollen seeds with shoot apex (bar = 1 mm). c. Asymbiotic seed germination in Murashige and Skoog (MS) medium plus 5 μM 6-benzyl aminopurine (BAP) (bar = 1 mm). d. Multiple shoot and root in MS plus 5 μM BAP and 15 μM indole 3-acetic acid (IAA) (bar = 1 mm). e. Seedling growth after 3 months in culture (bar = 2.5 cm). f. Hardened plants of *Vanda coerulea* (bar = 2.5 cm).



Seeni and Latha, 2000). Therefore, there is an urgent need to conserve this rare and endangered taxon.

Earlier reports on embryo culture and *in vitro* multiplication of *V. coerulea* have been confined to results obtained on Vacin and Went (1949) medium (Nath et al., 1991; Devi et al., 1998; Seeni and Latha, 2000; Malabadi et al., 2004). Responses of seeds on other nutrient media have not been encouraging (Roy et al., 2011). According to Roy et al. (2011), the highest seed germination of 62.33% for *V. coerulea* was achieved on Phytamax basal medium. Therefore, the main objective of this study was to assess the suitability of different nutrient media and growth regulators for improving the propagation of *V. coerulea*, a rare and endangered orchid species.

MATERIALS AND METHODS

Plants of *V. coerulea* collected from the forests of Meghalaya, India, were maintained in the glasshouse of the Botany Department, North-Eastern Hill University, Shillong, India. Approximately 10 to 11-month old unopened hand pollinated capsules were harvested from the plants. The collected capsules were scrubbed with a soft brush, washed thoroughly with detergent under tap water and surface sterilized in 70% ethanol for 2 min followed by surface flaming. This process was repeated three times, after which the capsules were rinsed four to five times with sterile distilled water. The surface sterilized capsules were then dissected longitudinally with a sterile scalpel. Around 100 mg of the powdery seeds were then scooped out and inoculated on different culture media in 25 × 150 mm glass test tubes, each containing 15 ml of medium.

Tested media included MS (Murashige and Skoog, 1962), Gamborg's B_5 (Gamborg et al., 1968), Mitra (Mitra et al., 1976) and Knudson C (Knudson, 1946). These media were incorporated with 3% (w/v) sucrose and solidified with 0.8% (w/v) agar. The pH of the media was adjusted to 5.8 ± 0.02 using 1N NaOH prior to autoclaving at 121 °C. Two growth regulators, indole 3-acetic acid (IAA) and 6-benzyl aminopurine (BAP), at concentrations of 5, 15, 30 and 50 μM, were singly added to the MS media to test their effects on seed germination. Each treatment consisted of 10 replicates and the experiments were repeated thrice. Cultures were incubated at 25 ± 2 °C under a daily 12-h photoperiod of 50 $\mu mol\ m^{-2}\ s^{-1}$ light intensity. Seeds cultured on various media were observed regularly for germination. Seeds were considered germinated upon emergence of the embryo from the testa (Kumaria and Tandon, 1991).

Three-month old seedlings measuring 0.5–0.7 cm in length (bearing two leaf initials without any roots) were used to test the separate and combined effect of growth regulators (BAP and IAA in MS medium) on growth and development. The growth regulators were added in the same concentrations listed above. Data on seedling growth parameters, namely shoot number, shoot length, root number and root length, was recorded after six weeks of culture. The experiments were repeated twice with five replicates per treatment. Seedlings with well defined roots (2–3 cm in length) were transplanted into clean thermocol pots, 8 cm in diameter, containing different compost mixtures. Mixtures included (i) brick pieces and charcoal chunks (1:1), (ii) brick pieces and charcoal chunks (1:1) plus a top layer of moss, (iii) brick pieces, charcoal chunks and decaying litter (1:1:1), and (iv) brick pieces, charcoal chunks and decaying litter (1:1:1) plus a top layer of moss. Transplanted seedlings were also fed fortnightly with diluted (10 times) MS nutrient salt solution. The survivability of the transferred plantlets was recorded after six weeks of transfer.

Data were subjected to one way analysis of variance and comparisons among treatment means were made using Fisher's LSD test.

TABLE 1. Effect of four different media, Murashige and Skoog (MS), Gamborg's B_5 (B_5), Mitra and Knudson C (KC), on seed germination (mean ± SE) of *Vanda coerulea* four weeks after planting.

Media	Germination (%)
MS	71.8 ± 0.37[a†]
B_5	53.4 ± 0.50[b]
Mitra	40.6 ± 0.50[c]
KC	34.2 ± 0.37[d]

[†]Means followed by the same letter are not significantly different according to Fisher's LSD test ($p \leq 0.05$).

RESULTS AND DISCUSSION

A high degree of variation was observed in percentage germination among different media (Table 1). The highest germination (71.8%) was recorded for seeds on MS medium (Fig. 1b). Seeds responded poorly to KC medium with only 34.2% germination. Further growth and development of germinating seeds was also optimal on MS medium. Asymbiotic seed germination has emerged as an important tool for propagating a large number of orchid species and hybrids (Arditti et al., 1982). However, germination of orchid seeds is difficult, as specific nutritional and environmental conditions are needed since seeds lack endosperm and cotyledons. Lipid droplets are the only seed storage materials localized within the embryo proper (Arditti and Ernst, 1984). These lipids are utilized during seed germination (Manning and Van Staden, 1987) and cytokinin is needed for lipid mobilization. *In vitro* growth and development is largely due to the composition of nutrient media which differ in the quantity of various salts and ions. Of all the mineral nutrients, nitrogen has been shown to have the highest impact on seedling growth and development, and nitrogen demand was reported to be closely related to the amount of growth and differentiation (Kramer and Kozlowski, 1979). In the present study and among the tested media, MS was found to be the best for seed germination and differentiation, confirming results of earlier studies (Reddy et al., 1992; Hoshi et al., 1994). Liu and Zhang (1998) found that ½ MS basal medium was best for embryo germination and proliferation of *Dendrobium candidum* Wall. Ex. Lindl. Presence of high N_2 content in the form of ammonium nitrate in MS medium might have promoted growth, with NH_4^+ and NO_3^- ions being readily assimilated during the initial and later stages of development, respectively. Some ammonic acids have been reported to replace ammonium nitrate in orchid culture (Van Waes and Debergh, 1986), which would explain the relatively good growth of seedlings on Gamborg's B_5 medium containing glycine that could have provided nitrogen for seedling growth. The present study showed that seed germination was reduced in Knudson C medium, probably

Table 2. Effect of varying levels of two growth regulators, 6-benzyl aminopurine (BAP) and indole 3-acetic acid (IAA), in Murashige and Skoog medium, on seed germination (mean ± SE), embryo emergence and protocorm development of *Vanda coerulea*.

Growth regulator	Concentration	Germination	Time taken in weeks for: Emergence of the embryo from the testa	Time taken in weeks for: Protocorm development with shoot apex
	(μM)	(%)		
BAP	05.0	94.4 ± 0.40[a†]	2	3
	15.0	90.6 ± 0.50[b]	2	3
	30.0	80.6 ± 0.50[c]	2	3
	50.0	—	—	—
IAA	05.0	92.6 ± 0.50[a]	2	3
	15.0	82.6 ± 0.50[b]	2	3
	30.0	74.6 ± 0.50[c]	3	4
	50.0	—	—	—

[†]Means followed by the same letter are not significantly different according to Fisher's LSD test ($p \leq 0.05$).

due to the presence of calcium in the form of $CaCO_3$. Alan (1989) reported that while calcium nitrate generally increased the availability of minerals, it reduced nitrogen availability to tissues.

Since the MS medium resulted in the highest germination percentage, it was selected for post-germination tests. Incorporation of either BAP or IAA singly in MS media further enhanced seed germination and seedling growth of *V. coerulea*, and all results were higher than those of seeds grown on basal MS with no BAP or IAA addition. A maximum germination of 94.4% and 92.6% (Table 2, Fig. 1c) was obtained on MS plus 5 μM BAP and MS plus 5 μM IAA, respectively. Shoot number was increased to 13.2 with an average 5.18 roots in MS medium supplemented with 5 μM BAP and 15 μM IAA (Table 3, Fig. 1d). Root length was highest (3.10 cm) at 5 μM of IAA. Growth regulators in the medium may act similarly to endophytic fungi, which bring about physiological changes in the developing orchid protocorms in nature (Kumaria et al, 1990; Kumaria and Tandon, 1994 and 2000). Incorporation of auxins in the medium has been reported to enhance seed germination and seedling growth of orchids. The germination frequency of *Dendrobium formosum* Roxb. ex Lindl. was enhanced with BAP additions (Dohling et al., 2008). Other studies have reported the stimulatory effect of BAP on protocorm multiplication and shoot formation in *Cymbidium pendulum* (Roxb.) Sw. (Pathak et al., 2001) and *Cattleya aurantiaca* (Bateman ex Lindl.) P.N. Don (Pierik and Steegmans, 1972). Combinations of auxins and cytokinins may enhance growth, but the effects of these combinations vary with the growth regulators used, their concentration, ratios and orchid species or hybrid. In this study, increased concentrations of

BAP had a negative influence on germination and growth, causing inhibition at high concentrations (Tables 2 and 3), confirming earlier findings by Nayak et al. (1997) and Nagaraju and Parthasarathy (1999). Auxins have been reported to enhance root formation in plants (Bhojwani and Razdan, 1983). In the present study, a significant and negative effect on root number was observed in the medium supplemented with IAA.

Different combinations of compost and seedlings with well defined roots (Fig. 1e) were used for hardening and establishment of seedlings grown *in vitro*. The survivability and growth of the seedlings was best in the potting medium comprised of charcoal, brick pieces and decaying litter (1:1:1) with a top layer of moss (Table 4, Fig. 1f), followed by charcoal, brick pieces and decaying litter (1:1:1). Successful transplanting of plantlets depends on size, seedling growth and the compost used. Hardy and vigorous seedlings are easier to transfer and are less susceptible to diseases and mechanical injuries than less hardy, weak seedlings. The transferred seedlings had a healthy and vigorous growing root system which ensured higher establishment and growth. Compost made up of charcoal, brick pieces, decaying litter and a top layer of moss produced the best seedling growth, probably due to its superior drainage and aeration for root respiration. The layer of moss on top proved to be beneficial due to higher retention of moisture. Vanda is a rapid-growing "hungry" orchid and hence compost with plenty of organic matter is of great necessity. Feeding the seedlings with diluted MS nutrient salt solution fortnightly also contributed to healthy growth by providing essential nutrients to developing seedlings, as previously reported by Kumaria and Tandon (1994).

In conclusion, the MS medium followed by planting in a compost mixture of charcoal, brick pieces and decaying litter with a top layer of moss was the most effective combination for obtaining high seed germination, survivability and growth of this orchid species. Given the rare and endangered status of *V. coerulea* in the wild, and the critically threatened status of its natural habitat, this protocol of *in vitro* germination and development of healthy plantlets can be used both for restoring the dwindling populations in nature and mass propagation for ornamental and pharmaceutical purposes.

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Table 3. Effect of varying levels of two growth regulators, 6-benzyl aminopurine (BAP) and indole 3-acetic acid (IAA), in Murashige and Skoog medium, on growth (mean ± SE) of three-month-old *Vanda coerulea* seedlings measuring 0.5 to 0.7 cm in length (bearing two leaf initials without any root), six weeks after transfer.

Growth regulator(s)	Concentration	Mean shoot number	Mean shoot length	Mean root number	Mean root length
	(μM)		(cm)		(cm)
BAP	05.0	04.8 ± 0.37[a†]	02.18 ± 0.10[a]	03.4 ± 0.50[a]	01.48 ± 0.05[b]
	15.0	02.6 ± 0.24[b]	01.32 ± 0.13[b]	02.6 ± 0.24[b]	01.46 ± 0.05[b]
	30.0	02.4 ± 0.24[b]	01.12 ± 0.11[b]	02.4 ± 0.24[b]	01.24 ± 0.02[c]
	50.0	02.2 ± 0.20[b]	01.00 ± 0.07[c]	—	—
Control	0	02.0 ± 0.00[c]	01.18 ± 0.10[b]	02.4 ± 0.24[b]	02.12 ± 0.07[a]
IAA	5.0	03.6 ± 0.29[a]	02.46 ± 0.06[a]	04.2 ± 0.37[a]	03.10 ± 0.07[a]
	15.0	02.5 ± 0.24[b]	01.28 ± 0.08[b]	03.6 ± 0.50[b]	02.44 ± 0.05[b]
	30.0	02.4 ± 0.24[b]	01.04 ± 0.09[b]	02.6 ± 0.24[c]	02.02 ± 0.05[c]
	50.0	02.2 ± 0.20[b]	00.92 ± 0.08[c]	02.4 ± 0.24[c]	01.70 ± 0.04[d]
Control	0	02.0 ± 0.00[b]	01.18 ± 0.10[b]	02.4 ± 0.24[c]	02.12 ± 0.07[c]
BAP + IAA	05.0 + 05.0	02.4 ± 0.24[e]	02.50 ± 0.05[c]	03.8 ± 0.37[b]	02.38 ± 0.05[a]
	05.0 + 15.0	13.2 ± 3.03[a]	05.18 ± 0.06[a]	05.1 ± 0.37[a]	02.24 ± 0.02[a]
	05.0 + 30.0	09.8 ± 0.24[b]	03.22 ± 0.03[b]	03.6 ± 0.50[b]	02.06 ± 0.02[b]
	15.0 + 05.0	05.8 ± 0.37[c]	02.02 ± 0.05[c]	03.2 ± 0.37[c]	01.76 ± 0.05[c]
	15.0 + 15.0	03.4 ± 0.24[d]	01.88 ± 0.03[d]	02.6 ± 0.24[d]	01.54 ± 0.05[c]
	30.0 + 05.0	02.2 ± 0.20[e]	01.58 ± 0.03[d]	02.4 ± 0.24[d]	01.34 ± 0.05[d]
	30.0 + 15.0	01.2 ± 0.37[e]	01.18 ± 0.10[e]	01.8 ± 0.37[e]	01.06 ± 0.04[d]
Control	0 + 0	02.0 ± 0.00[e]	01.18 ± 0.10[e]	02.4 ± 0.24[d]	02.12 ± 0.07[b]

[†]Means in each column, within a group of growth regulators, followed by the same letter, are not significantly different according to Fisher's LSD test ($p \leq 0.05$).

Table 4. *Ex vitro* establishment (mean ± SE) of *Vanda coerulea* plantlets six weeks after transfer to different potting media.

Potting mixture	Survival	Plantlet height
	(%)	(cm)
Brick pieces and charcoal chunks (1:1)	51.2 ± 0.58[d†]	3.60 ± 0.07[d]
Brick pieces and charcoal chunks (1:1) plus a top layer of moss	56.0 ± 0.44[c]	4.16 ± 0.05[c]
Brick pieces, charcoal chunks and decaying litter (1:1:1)	85.8 ± 0.37[b]	5.70 ± 0.07[b]
Brick pieces, charcoal chunks and decaying litter (1:1:1) plus a top layer of moss	91.2 ± 0.58[a]	7.22 ± 0.10[a]

[†]Means in each column, followed by the same letter, are not significantly different according to Fisher's LSD test ($p \leq 0.05$).

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