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In vitro multiplication and ecorehabilitation of the endangered Blue Vanda

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Abstract

Rapid multiplication of endangered *Vanda coerulea* Griff ex. Lindl. (Orchidaceae) was achieved through culture of shoot tips of mature plants, shoot tips and leaf bases of 8-month-old axenic seedlings on Mitra et al. (1976) medium supplemented with 10% coconut water, 500 mg l^{-1} casein hydrolysate and a combination of 8.8 μM benzyladenine and 4.1 μM napthaleneacetic acid (NAA). Shoot tips (0.5 – 0.8 cm) cultured in both agar and agitated liquid media responded alike with direct formation of 3–12 shoot buds in 12 weeks, while callus-free formation of 3–8 protocorm-like bodies (PLBs) in 4–8 weeks was noticed in seedling leaf base segments (1.0–1.5 cm). Formation of new buds or PLBs, rapid growth of buds into shoots and emergence of shoots from PLBs occurred when the explants with proliferating buds / PLBs were subcultured in medium enriched with 35 g l^{-1} banana pulp, 30% coconut water and 1.08 μM NAA. A maximum of 70 and 100 shoots of varied length were obtained from a single shoot tip/leaf base explant after 30 weeks of culture. Rooting of shoots (3.0–4.0 cm) occurred in medium containing 35 g l^{-1} banana pulp and 1.08 μM NAA within 3–4 weeks. Rooted plants were established easily (95–100%) in community pots without hardening. The regenerated plants were cytologically stable ($2n$ = 38). They were free from morphological, growth and flowering abnormalities. Eighty-five community potted plants were reintroduced into alien forest habitats at Ponmudi and Palode in southern ranges of the Western Ghats. They established at 70–80% rate.

Abbreviations: BA – 6-benzyladenine; BP – banana pulp; CH – casein acid hydrolysate; CW – coconut water; NAA – naphthaleneacetic acid; PLBs – protocorm-like bodies

Introduction

Vanda coerulea Griff ex. Lindl. (Orchidaceae), popularly known as the Blue Vanda of Asia, is a perennial epiphyte growing at elevations of 1000–1500 m in the Khasia and Jaintia Hills of the state of Meghalaya in India and in the northern ranges of Thailand and Burma. Acclaimed as the 'Queen of the Vandas' (Grove, 1984), it blooms two to five times a year in its natural habitat and in cultivation. It has been bred for such qualities as flower size, floriferousness, vigour and cold tolerance in modern vandaceous hybrids (Motes, 1988). Habitat destruction and overexploitation are the two important factors threatening its survival in India (Pradhan, 1985). Appendix I, formulated by the Committee for International Trade in Endangered Species of Wild Fauna and Flora, prevents its trade. It is also included in the Threatened Plants List of India published by the International Union for Conservation of Nature and Natural Resources. There is an urgent need to conserve this rare taxon.

Plant tissue culture is a potentially useful technique for *ex situ* multiplication and restoration of ill-fated taxa (Wochok, 1981). In the present paper, we describe rapid multiplication of *Vanda coerulea* through shoot tip and leaf base culture and successful establishment of the clonal plants in forest segments of south Western Ghats of peninsular India.

Materials and methods

Plant materials

Twenty mature flowering plants of *V. coerulea* were obtained from nursery collections at Shillong. The plants were established in pots.

Top shoot cuttings, each with 4–5 nodes collected from mother plants were washed well in running tap water and surface decontaminated by successive immersion in 1% (v/v) Teepol (Central Drug House, New Delhi), 70% ethanol (20 sec) and 0.1% $HgCl_2$ solution (3 min) and washed thrice with sterile distilled water. Shoot tips measuring 0.5 – 0.8 cm and the youngest two leaves dissected out of the plants were used as explants.

Seeds from the disinfected green pods (4 months after self-pollination) were sown on Knudson C agar medium (Knudson, 1946), supplemented with 2% sucrose and 10% coconut water. Six-month-old seedlings served as the source of leaf and shoot tip explants used in culture experiments.

Culture initiation

Leaves, leaf bases (0.9 – 1.5 cm) and shoot tips of both mature plants and axenic seedlings were implanted vertically, 2–3 mm deep in culture initiation medium consisting of the salts and vitamins of Mitra et al. (1976), 3% sucrose, 10% CW and 500 mg l^{-1} peptone. Plant growth regulators (BA and NAA) were incorporated into the medium at varying concentrations and combinations. The pH of the medium was adjusted to 5.2 before adding 0.7% agar (Oxoid Limited, UK) and autoclaved at 121 °C and 108 kPA for 18 min. All the cultures were incubated at 24 ± 2 °C under 12-h photoperiod at a photon flux density of 20–50 μ mol m^{-2} s^{-1} from daylight fluorescent tubes (Philips India Ltd., Bombay). Each treatment consisted of 20–25 explants in the case of seedlings. Owing to the limited number of mature plants, only a few treatments, each with 5 explants, could be tried. In certain cases, liquid culture of the shoot tips and leaf bases (1–2 explants in 50 ml medium in 100 ml Erlenmeyer flasks) was also tried under constant agitation at 70 rpm on a New Brunswick G-10 gyrotory shaker.

Multiplication and rooting

After 8–12 weeks, the shoot tips, leaves and leaf bases with differentiated PLBs or proliferating shoot buds were subcultured in the same medium with or without enrichment with 35 g l^{-1} ripe BP, 30% CW and 1.08 μM NAA. Healthy shoots having 3–4 leaves were obtained within 8–12 weeks. In order to increase the stock of shoot cultures, young leaf bases excised from these shoots were subcultured into the initiation medium to produce fresh crops of PLBs. For rooting, the shoots were transferred to an enriched medium (35% BP and 1.08 μM NAA) devoid of CW.

Field establishment and ecorehabilitation

The rooted plants, removed from the flasks were washed free of agar in running tap water and transplanted into 5-cm dia. clay pots in a potting mixture of charcoal chips and broken tiles (2:1). Two or three plants were planted in each pot in such a way that the roots passed downwards touching the charcoal and tile pieces. The plants were watered twice daily and fertilized at weekly intervals with a foliar spray of Vijay Complex, NPK 17:17:17 (Madras Fertilizers Co., Madras). After 6 months, the rooted plants were individually transferred to 20-cm pots and watering and nutrient schedules continued.

Twenty-five well established 3-month-old community potted plants were reintroduced in late May into a natural shola forest of Ponmudi in Thiruvananthapuram District of Kerala, 1000 m above mean sea level. The temperature in this region ranged from 7–30 °C. The host trees selected were *Maesa perettetiana* (Myrsinaceae), *Clerodendron* species (Verbenaceae) and *Machilus macrantha* (Lauraceae). Sixty plants were also reintroduced into natural forest segments in the Institute's campus at Palode (150 m) where relatively warm (22–34 °C) and humid conditions prevailed.

Plants with the roots still attached to or detached from the charcoal chips were tied on to the trunk and branches of the trees at various heights using nylon thread. Loose coconut husk or moss fibres were gently woven around the roots of some plants before tying them on to the trees. No attempt was made to water or fertilize the plants as pre-monsoon showers in May bathed them at regular intervals. Monitoring of the establishment was done at monthly intervals.

Histological studies

For histological studies, the tissues were fixed in formalin–acetic acid-alcohol (FAA 1:1:18) for one week, dehydrated in t-butyl alcohol series and embedded in paraffin (Johansen, 1940). Sections were cut

Table 1. Shoot bud formation in shoot tip explants from axenic seedlings and mature plants of *Vanda coerulea* cultured in liquid and agar modified Mitra et al. medium containing 3% sucrose, BA (8.8 μM) and NAA (4.1 μM)

Culture medium	Source	Number of shoot tips cultured	Shoot tips producing Shoot buds (%)	Mean number of shoot buds / explant
Liquid	Axenic seedlings	20	15(75.0)	4.2[a]
	Flowering plants	5	3(60.0)	9.3[b]
Agar	Axenic seedlings	15	9(60.0)	3.1[c]
	Flowering plants	5	2(40.0)	12.5[d]

Flowering plant-derived shoot tips were subcultured in fresh media at 4 week intervals. Observations were made after 12 weeks of culture.
Means followed by different letters are significantly different ($p < 0.05$) based on L.S.D. multiple range test.

at 7–10 μm, stained with safranin – fast green and mounted in Canada balsam.

Cytological studies

Root tips of the randomly selected, 7–8 month old community potted plants were transferred to a few drops of lactopropionic orcein (lactic acid : propionic acid: orcein 5 : 5 : 2), placed on clean microslides, softened by boiling over a spirit lamp and squashed under coverslips to yield a thin spread of cells for chromosome counts under a Leitz Orthoplan microscope.

Results

The morphogenetic responses differed among the explant types and sources of explants cultured in the initiation medium. Shoot tip cultures initiated from mature plants and axenic seedlings readily produced buds in both liquid and solid media in 10–12 weeks. The media turned brown owing to release of exudates from the cut ends which necessitated the transfer of the mature plant-derived shoot tips to fresh medium at 4 week intervals. At the end of third transfer (12 weeks), 40–75% of the shoot tips had formed 3–12 buds (Table 1, Figure 1a). Although somewhat higher percentage of seedling-derived shoot tips responded well for bud initiation, the number of buds (9–12) differentiated upon the shoot tips of mature plant origin were significantly high. However, discrete PLBs were not formed in both the sources of shoot tips.

Table 2. Formation of PLBs from the base of different leaves of *V. coerulea* seedlings. Five-month-old seedlings raised from embryo cultures consisted of 5–6 leaves

Leaf position*	Number of leaves cultured	Number of leaves forming PLBs (%)	Mean number of PLBs/leaf**
1	25	22(88)	11.2[c]
2	26	25(96)	13.6[d]
3	26	22(85)	12.4[c]
4	26	21(81)	9.7[b]
5	22	14(64)	7.1[a]

The leaves of different positions were cultured for 12 weeks with their bases embedded in modified Mitra et al. agar medium containing 3% sucrose, 8.8 μM BA and 4.1 μM NAA.
*Leaf position numbered basipetally from apex.
**Means followed by the same letter are not significantly different ($p < 0.05$) based on L.S.D. multiple range test.

Basal parts of the top two leaves of mature plants swelled in size followed by minor callusing for 4–6 weeks but did not form PLBs or buds. After 10–12 weeks, the tissues embedded in the medium turned pale brown while the medium itself was free from exudates. Transfer to fresh medium did not reverse the eventual degeneration of the tissues.

Formation of PLBs was distinctly noticed in seedling leaf cultures. Approximately 60% of the seedling leaf bases cultured in liquid medium yielded 4–6 PLBs in 4 weeks. In agar media, 64–96% of the isolated leaf bases and basal parts of the intact seedling leaves embedded in the medium responded with the formation of 3–8 PLBs in 6–8 weeks (Figure 1b). Although all the leaves of the seedlings responded positively to shoot bud formation via. PLBs, the degree of response declined as the age of the leaf increased. Thus, the fifth leaf from above responded less frequently (64%) with fewer PLBs (7.1) compared to the second leaf (96%) with 13.6 PLBs (Table 2). Within 12 weeks of culture initiation, shoots had already emerged from some of the early formed PLBs.

The ratio of BA and NAA in the medium greatly affected PLB formation and shoot differentiation (Table 3). When the leaf bases of seedlings were cultured in the presence of BA alone, 1–3 shoots were formed in approximately 20% of the explants in 12

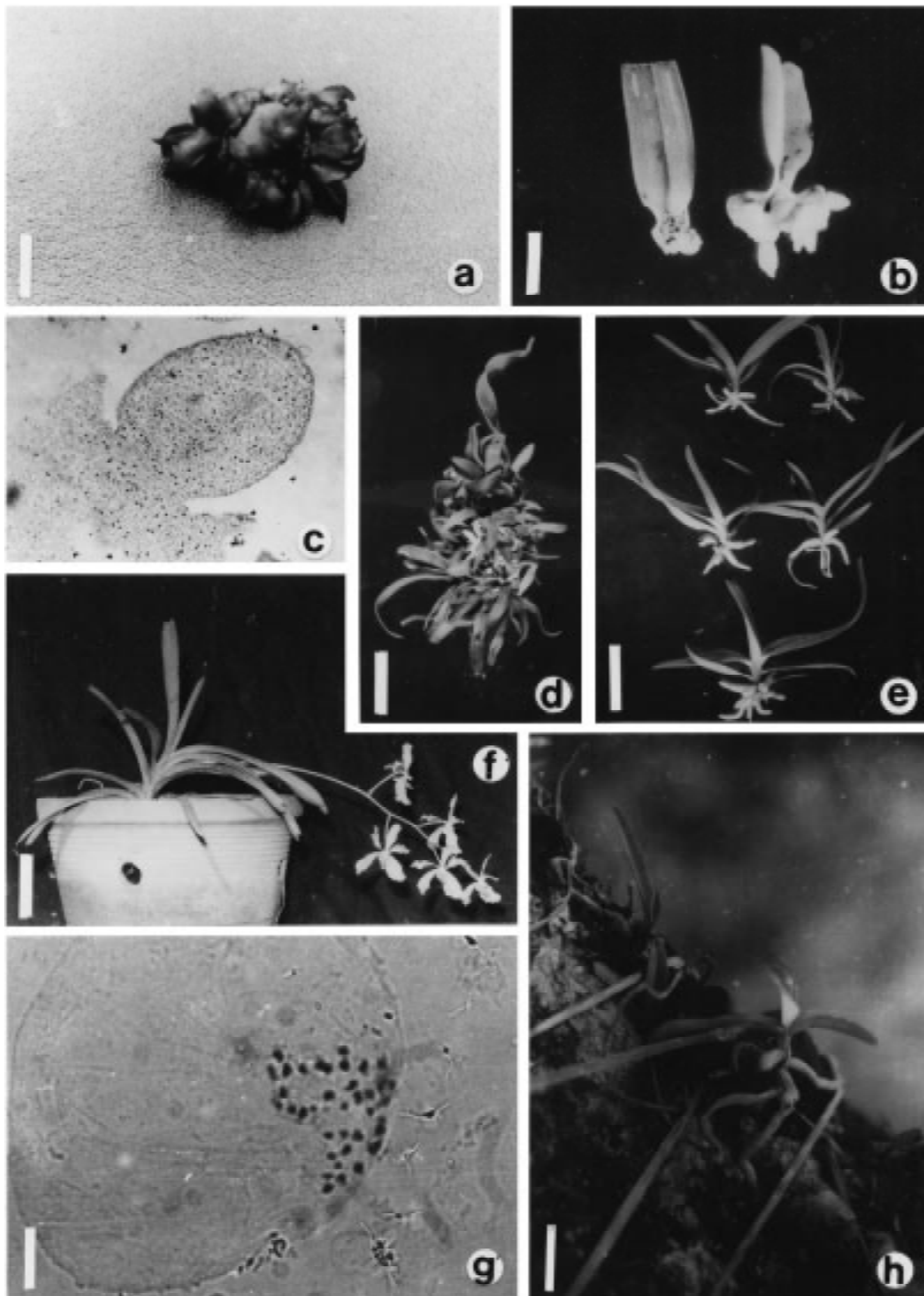

Figure 1. In vitro multiplication and ecorehabilitation of *Vanda coerulea* Griff. ex. Lindl. (*a*) Bud formation in shoot tip explant after 12 weeks of culture in modified Mitra et al. medium containing 8.8. μM BA and 4.1 μM NAA (Bar = 1 cm); (*b*) Protocorm-like bodies developed from leaf bases after 3 (L) and 6(R) weeks of culture (Bar = 1 cm); (*c*) Section of leaf base showing the epidermal and subepidermal origin of the ellipsoidal PLB (Bar = 0.05 cm); (*d*) Shoots proliferated upon the leaf base after 12 weeks of culture (Bar = 1 cm); (*e*) Rooted plants obtained in the enriched medium containing BP and NAA (Bar = 1 cm); (*f*) Micropropagated plant producing inflorescence, 36 months after planting (Bar = 4 cm); (*g*) Root tip cell showing 2n=38 chromosomes (Bar = 0.01 mm); (*h*) Establishment of micropropagated plants on bark of host *Syzygium* tree, 5 months after reintroduction (Bar = 2 cm).

Table 3. PLB formation and shoot regeneration from seedling leaves of *Vanda coerulea* cultured for 12 weeks in the initiation medium and subcultured for 8 weeks in the Mitra et al. medium containing 3% sucrose

Growth regulators		Number of leaves cultured	Number of leaves producing PLBs(%)	Mean number number of PLBs/leaf	Mean number of shoots / leaf
BA (μM)	NAA (μM)				
4.4	0	25	5(20)	10.2[a]	2.3[a]
0	2.7	20	2(10)	0	0
4.4	2.7	20	3(15)	25.1[b]	10.2[b]
8.8	2.7	25	7(28)	32.3[c]	15.2[c]
13.2	2.7	25	10(40)	38.2[d]	27.2[de]
4.4	4.1	24	6(25)	60.1[e]	30.2[e]
8.8	4.1	20	14(70)	75.3[f]	40.2 [f]
13.2	4.1	20	14(70)	63.3[e]	25.1[d]
4.4	5.4	25	15(60)	40.3[d]	27.8[de]
8.8	5.4	24	10(42)	27.1[b]	15.3[c]
13.2	5.4	25	10(40)	12.3[a]	4.1[a]

Concentrations and combinations of growth regulators used were the same in both initiation and subculture media.
Means followed by the same letters are not significantly different ($p < 0.05$), based on L.S.D. multiple range test.

weeks. A combination of 8.8 μM BA and 4.1 μM NAA induced higher frequency (92%) formation of maximum number of PLBs (10–15) and shoots (4–7) in 12 weeks. Thin sections of the leaf base revealed the epidermal and subepidermal origin of the globular to ellipsoidal PLBs, essentially made up of parenchymatous cells (Figure 1c). During subculture of the explants with the PLBs proliferated upon in the same media used for culture initiation, continued formation of PLBs was noticed only upto 8 weeks, by which time shoots were already differentiated from most of the preformed PLBs. Altogether, a subculture for 12 weeks resulted in the formation of more than 40 shoots from each leaf base (Figure 1d). However, when subcultured in an enriched medium containing 35g l^{-1} BP, 30% CW and 1.08 μM NAA, continuous production of PLBs with simultaneous differentiation of shoots from the early-formed PLBs occurred, resulting in the production of a heterogeneous population of shoots. Starting from culture initiation, as many as 100 shoots of varying size were regenerated from a single leaf after 30 weeks; whereas, shoot tips produced only 70 shoots. The use of enriched medium with BP supplementation for PLB initiation was disadvantageous as only 30% of the leaf bases produced PLBs, and the rest turned pale and necrosed.

On subculture in the initiation medium, the young leaf bases isolated from shoot cultures responded as effectively as the seedling leaves with the formation of 8–14 PLBs in 12 weeks and emergence of equal numbers of PLBs and shoots during subculture in the enriched medium. No obvious decline in the regeneration efficiency was noticed when the foliar tissues of shoots were repeatedly subcultured over a 24-month period. This provided an effective mechanism for the multiplication of the species on a continuous basis.

More than 95% of the shoots regenerated from leaf bases and shoot tips produced roots within 3–4 weeks when they were separated and planted in the enriched medium containing BP and NAA (Figure 1e). Root formation did not affect the growth of shoots to any extent. The robust plants with 4–5 leaves and 3–6 roots were removed from the flasks, transplanted and established in the community pots at a frequency of 95–100%. They responded well to the watering and nutrient schedules followed in the nursery. After replanting in 20-cm pots and attaining a height of 30 cm in 30–36 months, the micropropagated plants produced inflorescences with 6–8 azure blue flowers each (Figure 1f). The plants showed uniform morphological growth and flowering characteristics. Root tip squashes of the randomly selected micropropagated plants revealed a chromosome complement of $2n = 38$ (Figure 1g).

The limited number of community potted plants transferred onto the host trees survived and more

Table 4. Ecorehabilitation of micropropagated *Vanda coerulea* plants into forest segments of Ponmudi and Palode in south Western Ghats of Peninsular India

Number of micro-propagated plants introduced	Host tree	Binding medium	Number of plants established after 5 months
Ponmudi			
10	*Syzygium* sp.	Nil	10 (100)
5	*Maesa perettetiana*	Moss	4 (80)
5	*Clerodendron* sp.	Nil	3 (60)
5	*Machillus macrantha*	Coconut husk	4 (80)
Palode			
20	*Aporosa lindleyana*	Nil	15 (75)
20	*Terminalia paniculata*	Coconut husk	16 (80)
20	*Dillenia pentagyna*	Nil	14 (70)

Observations were made 5 months after introducing the plants.

than 85% established at Ponmudi and 70% at Palode (Table 4). The plantlets tied with the roots closely pressed to the surface of the trunk showed extensive growth of the existing roots and formation of new roots (Figure 1h). The growing and newly formed root tips were either green or light purple in colour and stuck firmly to the surface of the tree trunks, often within the crevices. Plantlets with damaged root tips showed poor establishment. No host tree specificity was noticed. Most of the introduced plants produced 1 or 2 new leaves within a 3–4 month period during the southwest monsoon (June–September). The plants that were not exposed to direct sunlight continued to show profuse growth even during summer while the directly exposed plants became stunted with root tips and leaves turning brown to varying extent.

Discussion

Successes achieved with the multiplication of tropical orchids of conservation and horticultural interest are generally through seed cultures (Fay, 1988). Though there are some reports on the successful multiplication of species orchids using tissue cultures (Dodson, 1981; Goh, 1990; Seeni and Latha, 1992), the results presented herein are new for *V. coerulea.* The ability to produce phenotypically uniform, cytologically stable plants in a callus-free state from the shoot tip cultures of flowering plants as well as foliar meristems of axenic seedlings carries considerable conservation significance for this endangered orchid.

Plant regeneration in tissue culture of orchids may occur via direct shoot bud formation or PLB mediation. However, organ specific formation of these regenerative structures viz shoot buds in shoot tips and PLBs in leaf bases irrespective of the source of explants (flowering plants and axenic seedlings) observed in *V. coerulea* is seldom reported. It appeared that the resident axillary meristems of the condensed nodes in the basal part of the shoot tips, relieved of the apical dominance by the defunct apical meristem, proliferated to produce 3–12 callus-free buds. On the other hand, the adventive meristematic cells spread all over the surface of the leaf base responded to favourable culture conditions initially by random mitotic divisions and then organization into PLBs before the emergence of shoot initials. The proximal part of the leaf in orchids is meristematic and, on excision and culture, differentiates into plantlets (Zimmer and Pieper, 1975). While better exposure to nutrients and growth regulators in the medium may result in the relatively high percentage response of the shoot tips cultured in liquid media, the significant differences in the number of buds formed between the two sources of shoot tip may be related to the size of the relevant meristems. Presumably the larger number of meristematic cells present in the axillary meristems of the larger – sized shoot tips of flowering plant origin contribute to an enhanced caulogenic response. However, Lakshmanan et al. (1995) developed a rapid regeneration system for *Aranda* ‘Deborah’ using thin shoot sections as explants.

Release of phenolic exudates into the medium in shoot tip cultures of orchids is known (Lim-Ho, 1981), especially when the explants are isolated from field-grown plants and quick transfer of the explants to fresh media as followed is often recommended to avoid possible inhibitory effects of the exudates (Compton and Preece, 1986).

Among the explants used, leaf bases from seedlings proliferated better and produced the maximum number of observed shoots. The higher regeneration ability of the young leaves of the seedlings in orchids is often attributed to the physiological age of the explant (Vij et al., 1984). That the basal part of the youngest leaf from flowering plants responded with mitotic divisions as evidenced from swelling and marginal callusing in 4–6 weeks but failed to produce PLBs or buds provided ample evidence for this kind of response. This was even further exemplified in the case of the axenic seedlings cultured under controlled conditions where progressive decline in the regenerative responses of the ageing leaves in basipetal order was noticed. However, this response may vary from species to species as in a related vandaceous species of India, *Renanthera imschootiana*, all three basipetal young leaves from the apex of the flowering plants responded favourably with direct shoot formation (Seeni and Latha, 1992).

Overall, the results obtained with the axenic seedlings (Table 2) and subsequently with the shoot cultures suggest that judicious exploitation of leaf base regeneration may eventually provide sustainable supply of planting materials for horticultural use and also guarantee the survival of this overexploited species in nature.

Although caulogenesis in tissue cultures of orchidaceous and non-orchidaceous taxa is a function of cytokinin activity, provision of cytokinin (BA) alone induced only marginal response in foliar tissue cultures of *V. coerulea*. The requirement of synergistic combinations of BA (8.8 μM) and NAA (4.1 μM) for maximum formation of PLBs and shoots is supported in the published reports on orchids (Kusumoto, 1979; Mathews and Rao, 1985). Also, the beneficial effects of such additives as CW, CH and peptone for bud and PLB formation have been long recognised. BP as a rich source of natural cytokinins inhibits culture initiation and promotes differentiation and growth of shoots at later stages (Withner, 1974; Arditti and Ernst, 1993). Accordingly, Valmayor et al. (1986) recommended the use of a less complex medium for initiation and addition of banana pulp after two months for multiplication of PLBs in meristem cultures of *Vanda*. This is further corroborated in the present study which suggests that supplementation of the medium with 3.5% BP may be beneficial to the multiplication of robust PLBs and early differentiation of shoots after an initial culture of the tissues for 12 weeks in medium devoid of BP.

Micropropagated plants transplanted in community pots were well acclimatized to the nursery conditions including the water and fertilizer schedules as evidenced from their high survival rate, growth of shoots and roots and flowering after 30–36 months. It should be noted that the *in vitro* derived plants may not preserve the entire intraspecific genetic diversity of *V. coerulea* as it occurs in nature. However, subsequent green pod cultures established from the micropropagated plants may help to produce some genetic diversity within the species.

The fact that 70–80% of the plants transferred from the community pots were rehabilitated and established with relative ease in alien forest segments, points to successful *ex situ* refuge and return back to nature of an orchid of great horticultural and conservation interest, perhaps for the first time in India. To date, only tissue cultured plants of *Epidendrum ilense* and *Bletia urbana* have been transferred to the wild (Christenson, 1989; Rubluo et al., 1989). It is also not uncommon that certain orchid species are introduced and naturalised across trans-national boundaries through conventional means (Obata, 1987; Haugli, 1987). As part of an efficient reintroduction strategy for epiphytic orchids, it is suggested that host trees with coarse bark are selected and the micropropagated orchids tied to the tree trunks with the roots closely adpressed to the surface of the bark. Also, the reintroduction trials should be conducted with well established seedlings during the early monsoon period to ensure the highest survival rate of the plants under the warm tropical conditions. The available data are insufficient to arrive at a conclusion regarding host specificity and binding medium requirements. Experiments are in progress to determine these requirements and also to monitor the reestablishment over prolonged periods.

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