A protocol for shoot multiplication from foliar meristem of

*Vanda spathulata* (L.) Spreng

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Leaf explants collected from flowering plants of *Vanda spathulata* were cultured in Mitra medium with combinations of 6-benzyladenine (BA; 13.2-88.8 μM) and indole-3-acetic acid (IAA; 0.0-85.6 μM). Combination of BA (66.6 μM) and IAA (28.5 μM) induced maximum shoots (17.33) from foliar meristems (leaf base). BA individually did not induce caulogenesis in leaf explants. For optimized multiplication, BA:IAA (2:1 μM) was essential at 22.2-88.8 μM of BA. Recultured leaf explants produced lesser number of shoots compared to original explants and were nearly equal at combinations of 22.2-44.4 μM of BA and 5.7-28.5 μM of IAA. Rooting of shoots (>95%) occurred in medium containing banana pulp (75 g/L) and IAA (5.7 μM) within 3-9 weeks. Plantlets with 2-5 roots of 2-5 cm length established easily in community pots at 80-90% rates without hardening.

**Keywords**: Foliar meristem, Micropropagation, Orchid, Shoot multiplication, *Vanda spathulata* L.

*Vanda spathulata* (L.) Spreng (Orchidaceae), is an important orchid with large golden yellow flowers, endemic to India and Sri Lanka. As a polyploid species (4x and 6x) with dominant gene(s) for yellow colour of flower, it served as a donor of yellow flower colour in hybridization between vandaceous orchids. Its distribution ranged from semi-arid habitat at sea level to scrub jungles of high ranges even exceeding 1000 m. Severe habitat destruction and consequently en bloc elimination of the species particularly in the plains coupled with indiscriminate collection resulted in its depletion in the wild. Hence, availability of the species from the wild for horticultural exploitation is not satisfactory. Conventional propagation through seed culture is less desirable due to long juvenile period of seed propagated plants before flowering. Thus, tissue culture method emerged as the preferred choice for the propagation of the species.

Hormonal requirement for regeneration from leaf, shoot tip or axillary meristems of a number of orchid taxa has been reported. Usually, combination of auxins and cytokinins are employed for multiplication in orchids. The hormonal requirement for various taxa to get higher multiplication varied considerably and this difference even exists between genotypes. Optimization of hormonal regime for micropropagation is thus essential for each species. Role of combination of BA and IAA for optimized multiplication from in vitro cultured nodes of *V. spathulata* have already been reported. The present work was extended the study to foliar meristems of *V. spathulata* and demonstrated the combinations and concentrations of BA and IAA suitable for continuous shoot multiplication.

Apical buds were collected from flowering plants, mature leaves were removed retaining three young terminal leaves, washed in running tap water, soaked in 2% (v/v) Labolene (Godrej, India) for 15 min and again washed in running tap water. The buds were disinfected by serial passage through 5% Sterilq (Combichem, Delhi) for 20 min, 0.1% HgCl2 for 12 min followed by three quick changes of sterile water. Three leaves (2-8 cm long) with their sheathing base were dissected out one by one and again disinfected in 0.1% HgCl2 for 5 min. The dead basal end of the leaf bases were trimmed off and implanted vertically (3-5 mm deep) in the medium supplemented with salts and vitamins and containing sucrose (20 g/L); agar (6 g/L), CDH, India); and plant growth regulators. Benzyladenine (BA; Sigma, USA) was used at concentrations of 13.3, 22.2, 44.4, 66.6, and 88.8 μM individually and in combination with indole-3-acetic acid (IAA; Sigma, USA) at 1.7, 2.8, 5.7, 17.1, 28.5, 40.0, 57.1 and 85.6 μM. The pH of the medium was adjusted to 5.6 before adding agar and autoclaved at
121°C under 104 Kpa for 18 min. All the cultures were incubated in a culture room maintained at 28°C ± 2°C and RH 50-60% under 12 hr photoperiod (illumination of 20-30 μmol m⁻² s⁻¹) provided with fluorescent lamps (Philips, India).

After three months of inoculation, the explants with buds proliferated upon were transferred to fresh medium of the same composition to promote their growth into shoots. For further multiplication, leaves (1-3 cm) excised from single axillary shoots raised in presence of BA (4.4-44.4 μM) were re-cultured in Mitra et al. medium containing BA (13.3-44.4 μM) and IAA (0.0-28.5 μM) and subcultured into fresh medium after 3 months. For rooting, shoots of 3-5 cm length were transferred to Mitra nutrient medium enriched with banana pulp (cv. Palayanthodan; 75.0 g l⁻¹) and IAA (5.7 μM). The rooted plants obtained after 45-60 days were transplanted into community pots.

Leaves at different maturity (three leaves from apical buds) were distributed equally to different treatment combinations. Due to limited number of plants available for the experiments, each treatment consisted of 8-10 explants and was repeated twice. Experiment on leaf re-culture was conducted thrice and each treatment consisted of 7-10 explants (1-3 terminal leaves). Data on number of shoots produced per explant were collected after 6 months of culture initiation and subjected to analysis of variance in a completely randomized model. The treatment effects were compared by LSD multiple t test (α = 0.05).

More than 80% of leaves were contamination free and 75% of them responded well. Shoot initials (Fig. 1a, b) were developed directly from the leaf bases after 45-60 days of inoculation preceded by swelling of bases and browning of the medium. Individually, BA (13.3-88.8 μM) did not induce bud formation from leaves. However, 22.2-88.8 μM of BA induced shoot buds in combination with IAA. A combination of 66.6 μM of BA and 28.5 μM of IAA induced maximum proliferation of 17.33 ± 5.7 shoots/leaf base. Other favourable combinations were more or less equally effective to induce satisfactory level of shoot multiplication (Fig. 2a). In spite of significant shoot bud initiation rates obtained, further development into shoots was poor at 22.2 μM of BA.

Re-cultured leaf bases showed initial swelling in 20 days followed by emergence of buds (Fig. 1c, d) after 45 days. A number of combinations of BA and IAA (Fig. 2b) were equally effective to induce multiple shoot formation and even at the best combinations (22.2 μM of BA + 5.7 and 17.1 μM of IAA) the number of shoots formed were significantly less, accounted for only 50% of the shoots formed during culture initiation. BA alone did not induce any morphogenetic response as in the case of mature plant derived foliar meristems.

Shoots raised in multiplication media were devoid of roots. But upon transplantation of individual shoots into banana pulp enriched (75 g l⁻¹) Mitra medium containing 5.7 μM of IAA, more than 95% of the shoots produced 2-5 roots of varying length (2-7 cm) within 45-60 days. The plants with 2-5 roots and 4-8 leaves were transplanted in community pots and established at a rate of 90-95% (Fig. 1e).

Among plant growth regulators, BA is probably the most widely used cytokinin for inducing caulogenesis in plant tissue cultures. Even individually it is known

![Fig. 1 — In vitro multiplication from foliar meristems of V. spathulata, cultured in Mitra medium. (a) Shoots initiated from leaf base after 120 days; (b) Multiple shoots developed from leaf base after 180 days of culture in medium containing BA (66.6 μM) and IAA (28.5 μM); (Bar = 1.25 cm); (c) Shoot buds developed from re-cultured leaf base after 60 days; (d) Shoots developed from re-cultured leaf base after 120 days of culture in medium containing BA (44.4 μM) and IAA (17.1 μM); (Bar = 1.0 cm); and (e) Rooted mericlones established in community pots (Bar = 2 cm).]
to induce multiple shoot formation in foliar meristems\(^{10,11}\), shoot tips\(^5\) and axillary buds\(^12\) cultures of orchids. Combination with auxin (NAA or IAA) further enhanced bud proliferation in shoot tip of *Cattleya*\(^3\), shoot tips and flower stalk buds of *Phalaenopsis* and *Doritaenopsis*\(^5\) and foliar meristems of *R. inschootiana* and *V. coerulea*\(^7,10\). In *V. spathulata* however, foliar meristems responded differently to 13.2-88.8 \(\mu\)M of BA tested individually and in conjunction with 0.0-85.6 \(\mu\)M of IAA.

Synergistic effect of cytokinins and auxins on shoot proliferation in tissue cultures of many angiosperms including orchids is well known\(^7,10,13,15\). Our experiments with leaf base cultures revealed combinations of BA and IAA induced significant shoot proliferation during culture initiation and multiplication stages. For optimum shoot proliferation, BA molarity should be more than twice that of IAA in the medium. Inhibition of bud formation in extremes of BA and IAA (Fig. 2a) indicated the requirement for a threshold level of cytokinin and balanced level of auxin for shoot initiation in unorganized adventitious meristems in leaf tissue.

Regeneration potential of explants from mature plant was complemented by significantly larger number of shoots harvested per explant (17.33) compared to *in vitro* explants (9.27) during same period (6 months) of culture. This difference may be related to different sizes of meristems present in two sources of explants as observed in shoot tip cultures of flowering plants and axenic seedlings of *V. coerulea*\(^10\). The isolated leaves of mature plants are larger in size than *in vitro* explants and therefore, the relative size of the meristems play an important role in caulogenesis. The proximal part of leaves meristematic in orchids\(^16\), in particular appears to have larger surface area as compared to *in vitro* plantlets. It is known that explants of shoot cultures in orchids\(^10\) having adapted to culture conditions, respond rapidly with the proliferation and early harvest of shoots. This was not the case in *V. spathulata* wherein the explants of adult plants were also equally juvenile and a 6-month period was needed to harvest 2-5 cm long shoots in both adult and *in vitro* explants during culture initiation. The present study provided an efficient and optimized foliar regeneration protocol for *V. spathulata*, a threatened floriferous orchid of Indian subcontinent.

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References


