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In vitro propagation of *Vanilla planifolia*, a tropical orchid

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A simple and efficient micropropagation protocol was developed for *Vanilla planifolia* using shoot tip and nodal segments as explants. The explants were cultured in MS basal medium with 1 mg/l BAP for 10 weeks and subcultured onto fresh medium every 4 weeks. The proliferating clusters were cultured in N69 basal medium with BAP (0.5 mg/l) + d-biotin (0.05 mg/l) + folic acid (0.5 mg/l) and 2% sucrose for elongation of shoots, formation of root initials and further proliferation of axillary shoots. Separation and culturing of elongated shoots in fresh medium for 2 to 3 weeks yielded 7 to 8 cm long plantlets, which were acclimatized in polytunnels. The clusters of shoots, which did not elongate, served as a continuous source of explants for multiplication.

VANILLA planifolia Andr. is an orchid, commercially cultivated for pods (beans) from which the popular flavouring substance called vanillin is extracted. Vanillin is mainly used in flavouring ice creams, soft drinks, condiments and oleoresins. The world production of vanilla beans is estimated to be about 3500 tonnes per annum and nearly 300 tonnes are used in USA alone. Malagasy Republic grows 70 to 80% of the world's vanilla crop. The world trade is around Rs 300 crores annually, amounting to nearly 7% of the total value of the spice trade[1].

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Traditionally vanilla is propagated from cuttings of mature vines and is raised in polybags. The method is not economical since the collection of stem cuttings leads to arrest of growth and development of the mother plant[2]. Moreover, the market demand for propagules is hardly met with such cuttings. As the growers are looking for alternate sources, micropropagated plantlets serve the purpose and are popularly used. Some protocols on micropropagation of vanilla have been reported using nodal segments[3–6] and aerial root tips[7,8], wherein shoot proliferation was achieved by axillary bud growth or protocorm formation. However, mass propagation for commercial cultivation requires a simple, economical, rapidly multiplying and highly reproducible protocol without an intervening callus or protocorm phase so as to give rise to true-to-type clones. We report here such a protocol for large-scale micropropagation of *Vanilla planifolia*.

Shoot tips and nodal buds excised from greenhouse-grown vines were used as explants. The explants were pre-treated with a solution of 0.2% Bavistin, 0.2% Streptocycline and 5 to 6 drops of liquid Dettol for 4 h, and washed with Tween-20 and sterile distilled water. The explants were surface sterilized with 0.1% $HgCl_2$ for 10 min, followed by several washes with sterile distilled water.

The explants were initially inoculated in V1, V2, V3 and V4 media (Table 1). They were incubated at 25 ± 1°C and a 12 h photoperiod with a light intensity of 2500 lux from cool, white, fluorescent tubes. The growing buds were transferred to the respective fresh medium after 4 weeks (Figure 1 *a*). The main bud was given a central longitudinal cut to injure meristem tissues. Each treatment consisted of 20 explants and was repeated thrice.

The explants in V1 medium were healthy and growing vigorously. Elongation of the bud was seen by 5 to 6 weeks (Figure 1 *b*). Induction of axillary proliferation occurred from the eighth week. The proliferating axillary buds were well-defined, pale green and 0.5 to 1 cm long with bulbous base and pointed tips. A two-fold increase in multiplication was seen by 8 to 10 weeks. Further transfer in the same medium resulted in proliferation of shoots in the two-fold ratio at every subculture cycle.

Table 1. Composition of the media used to culture *Vanilla planifolia*

Medium code	Medium composition (mg/l)
V 1	Modified MS* + BAP (0.5) + sucrose 3%
V 2	MS + BAP (1) + Sucrose 3%
V 3	N 69** + BAP (1) + biotin (0.05) + folic acid (0.5) + sucrose 2%
V 4	N 69 + BAP (0.5) + Biotin (0.05) + folic acid (0.5) + sucrose 2%

*Modification to MS[12] (Murashige and Skoog basal medium) with NH_4NO_3, 1200 mg/l and KNO_3, 2500 mg/l.
**Nitsch basal medium[13]; BAP, 6-benzylaminopurine.

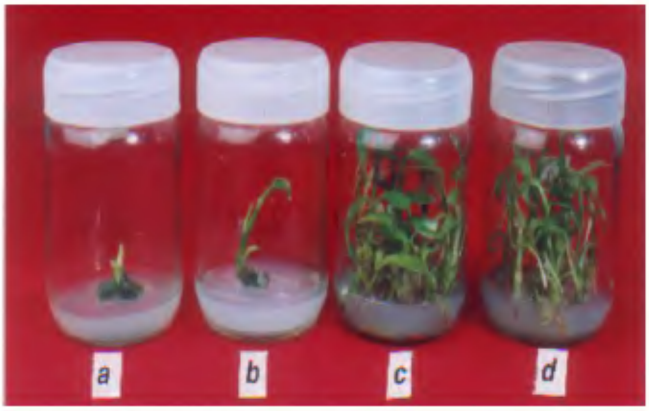

Figure 1. Response of vanilla explants to different culture media; ***a***, Explant transferred to V1 medium after 4 weeks of initiation; ***b***, Elongated bud vigorously growing in V1 medium; ***c***, Proliferating shoots being cultured in V4 medium in which a few elongated shoots can also be seen; ***d***, Elongated shoots separately being grown in fresh V4 medium.

In V2 medium, a two-fold increase in multiplication was observed in the second subculture, followed by three- to four-fold increase in the subsequent subcultures. Prolonged culture of multiplying clusters in V2 medium resulted in distortion of shoots. The shoots were highly stunted after the fourth subculture. Browning of the tips of proliferating shoots was also observed. Continuation of culturing beyond 16 weeks resulted in the growth of a compact and undifferentiated mass of callus with complete distortion of shoots.

In V3 medium, initially elongation of the bud was seen. The growing shoots consisted of broad leaves with 1 to 2 axillary buds arising from the base occasionally. The shoots were pale yellow and lanky. Hard mass of callus was seen in the third subculture without further proliferation of axillary shoots.

In V4 medium, explants elongated bearing broad leaves with slow axillary proliferation. A two-fold increase in axillary shoots was obtained in subsequent subcultures along with elongated shoots and root initials.

Based on these observations, the explants cultured for 12 weeks in V2 medium, which had attained the stage of vigorous proliferation, consisting of 6 to 8 shoots, were divided into smaller clumps. Each clump consisting of 3 to 4 shoots was transferred to V4 medium. In this medium, the dwarf shoots recovered to normal growth with more number of axillary shoots arising from the base. At the same time, 2 to 3 vigorously growing shoots from each clump elongated with expanded leaves by 4 weeks (Figure 1 *c*). Such shoots also had healthy, robust root initials. Subsequent transfers

into V4 medium gave rise to a three-fold increase in proliferating clumps, and 2 to 3 elongated shoots with root initials from each clump. The elongated shoots (4 to 5 cm long) were excised and cultured separately in fresh V4 medium (Figure 1 *d*) to encourage formation of long shoots, broad leaves and basal roots. The basal tuft of rooting was observed in more than 95% of the transferred shoots. The proliferating clumps were transferred to fresh V4 medium for further multiplication. The elongated shoots attained a length of 8 to 9 cm in about two weeks and were ready to be transplanted.

The plantlets were washed thoroughly with water, planted in Soilrite, a commercially available sterile potting mix, in net pots and hardened in a polytunnel under 100% relative humidity (RH) for 15–20 days. They were gradually exposed to decreasing RH and were brought out of the tunnel after 5–6 weeks. A survival rate of 88–94% was achieved during the hardening of the plants. The plants were further grown in shade until final despatch.

It is evident from the results that vanilla can be propagated *in vitro* using shoot tip and nodal buds as explants. Multiple shoot regeneration has been reported[6] using axillary nodal buds, cultured in MS + IAA (2 mg/l) + kinetin (0.5 mg/l) + BAP (0.5 mg/l) + biotin (0.2 mg/l) + Ca-pantothenate (0.2 mg/l), wherein a maximum of 6 shoots per explant, 2 cm long were obtained. However, these shoots had to be further cultured in MS + NAA (0.5 mg/l) + kinetin (0.1 mg/l) to obtain rooted plantlets measuring 4 to 4.5 cm length.

It seems that for vanilla, a basal medium consisting of high salt concentration such as MS is essential in the first stage of culture. In all the previous reports on vanilla micropropagation, full strength MS basal had been used[5–8]. Though Philip and Nainar[7] have used four different basal media, viz. Knudson C, MS, Gamborg's and SH to culture stem sections and root tips, MS alone has been reported to be suitable for optimal plantlet induction and sustained growth.

It is observed in the present study that the axillary proliferation is initiated only when the shoot tip is injured by giving a longitudinal bisection. This might be due to the strong apical dominance exerted by shoot tip meristem with the consequent inhibition of axillary buds, as has been previously reported by Lakshmanan *et al.*[9].

Use of organic substances like d-biotin and Ca-pantothenate has been reported to enhance multiplication in vanilla[6]. During the present investigation, although no comparative studies were made on addition of adjuvants, it is assumed that the presence of d-biotin and folic acid in V4 medium might be one of the reasons for obtaining a continued proliferation of axillary shoots and also elongation of shoots with root initials, forming complete plantlets.

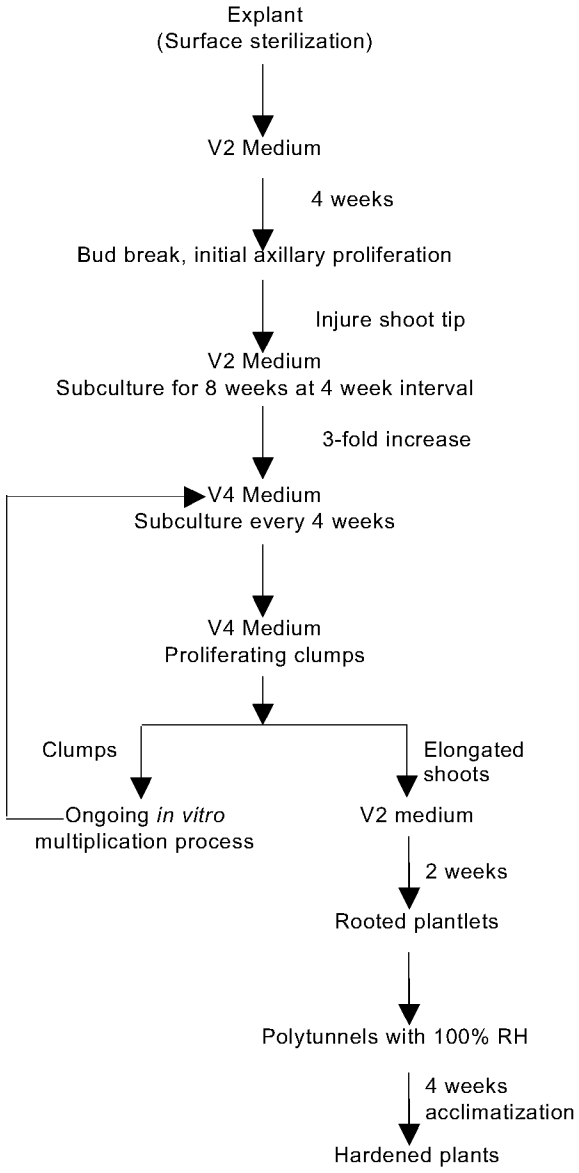

Figure 2. *In vitro* protocol for micropropagation of *Vanilla planifolia*.

The present study has shown that after obtaining substantial proliferation (8 to 10 shoots per explant per subculture), transfer of multiplying clusters to a basal medium with lower salt concentration like that of N69, lower BAP (0.5 mg/l) and lower sucrose (2%), is essential for conditioning the cultures of vanilla, as seen in *Tagetes*[10]. In N69 basal, the growing clumps not only proliferated but also gave rise to elongated shoots with broader leaves and root initials. A similar observation has been made in *Ixora singaporensis*[11], in which roots were induced by transferring the shoots previously cultured in MS basal to B5, a low-salt medium.

In conclusion, we have developed a micropropagation protocol for vanilla (Figure 2), which includes two me-

dia. The first one is V2 [MS + BAP (1 mg/l) + sucrose (3%)] in which the explants are initiated and cultured for 3 to 4 months at a 4-week subculture cycle. The second one is V4 [N69 + BAP (0.5 mg/l) + biotin (0.05 mg/l) + folic acid (0.5 mg/l) + sucrose 2%] in which proliferating shoots are obtained. From these proliferating shoots, supply of both rooted plantlets and proliferating clusters of shoots are available continuously, which is an essential requirement for mass propagation. The number of plants produced during the past three years up to December 1999 is 1,78,000. The number of plants supplied to the farmers between 1997 and 1999 is 1,60,000. The plants that have been supplied to coastal Karnataka are yielding now.

By the end of five subcultures, more than 250 shoot buds are obtained from a single explant, which when transferred to V4 medium can give rise to ca. 50 plantlets measuring 7 to 8 cm. Subsequently, the remaining 200 shoot buds which are in active proliferation, result in two- to three-fold increase in multiplication ratio, besides simultaneously generating rooted plantlets in an increasing order of 50, 100, 200, 400 and so on each subculture cycle. Thus, from a single explant it is possible to regenerate as many as one lakh plants in about 15 subcultures.

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