

In Vitro Propagation of *Vanilla Planifolia* Andr. -A Spice Orchid



Agriculture

KEYWORDS : In vitro, orchidaceae, stem-node, *Vanilla planifolia*, spice orchid

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ABSTRACT

This communication reports simple and effective *in vitro* protocol for *in vitro* propagation of *Vanilla planifolia* using uni-nodal stem segments in Mitra (M) medium and Murashige & Skoog (MS) medium alone and their combinations with cytokinins such as BA, Kn and auxin; NAA (1.0, 2.0 mg/l) each. The regeneration response was obligatory to use of growth adjuncts in M medium. 100% explants responded in both (M and MS) medium supplemented with BA (2 mg/l) and a maximum of 6.0 ± 0.81 and 6.0 ± 0.86 shoots were obtained in M and MS medium respectively using BA (2 mg/l). M medium with BA (2 mg/l) favoured early plantlet development within 11.65 ± 0.41 weeks. Extra axillary meristematic loci were induced opposite to the dormant axillary bud position in M+BA (1 mg/l) supplemented medium. Higher concentration of BA (2 mg/l) in the cultures allowed multiplication of shoot bud. NAA (1, 2 mg/l) favoured callusing at basal part of shoot bud and initiating multiple shoot buds. The regenerated shoots rooted in their respective media combination developing into complete plantlets.

Introduction:

Vanilla planifolia Andr. (= *Vanilla fragrans* Salisb.) is a tropical, herbaceous, perennial climbing orchid which possess greenish-yellow flowers. *Vanilla* is indigenous to south-eastern Mexico. It is the second most expensive spice after saffron and earns heavy amount of money (Ranadive 1994). *Vanilla planifolia* known as 'green gold' is the most famous commercial crop world over. It is the source of natural vanillin, which is a major component of flavour industry (Goodenough 1982). The world-wide production of vanilla is estimated to be about 3,500 tonnes per annum and 70 to 80% of the world's vanilla crop is grown by Malagasy Republic (Geetha and Shetty, 2000).

Besides having great economic value, it is known for its medicinal properties as well. The extract of this plant is useful in treating hysteria, rheumatism and other low forms of fever. The principal constituent of *Vanilla planifolia* is vanillin, chemically known as methylprotocatechuic aldehyde. It is used to flavour medicinal syrups. In addition, this orchid contains alkaloids, flavonoids, glycosides, carbohydrates and other phytochemicals. All the parts of this plant, viz., leaves, and stem possess some measurable inhibitory action against the pathogens (Shanmugavalli *et al.*, 2009).

V. planifolia is a threatened species. Major causes which account for the destruction of its natural habitats are the deforestation and over collection for trade and commerce. Conventional propagation method by stem-cuttings is time consuming and being monopodial cause injury to the mother plant. Besides this, there is possibility of perpetuation of certain viral infections with this method. *In vitro* culture techniques offer a viable system for true-to-type rapid mass multiplication and germplasm conservation of rare, endangered, aromatic and medicinal plants (Karuppusamy and Pullaiah, 2007, Mallon *et al.*, 2010). Through tissue culture techniques, a large number of true-to-type, pathogen-free plantlets can be effectively developed in short span of time.

In clonal propagation, it is immensely important to maintain genetic uniformity of *in vitro* raised progenies. In outbreeding taxa like orchids, seed raised progenies are extremely heterozygous. To maintain genetic purity of the regenerants, an appropriate *in vitro* propagation protocols should be devised. An effective alternative to shoot meristem for micropropagating orchids is *in vitro* culture of stem-node segments. This method provides opportunities to produce a large number of true-to-type plantlets of interest. The regenerative competence of stem-node segments are tested in orchid species of diverse habit and habitats (Arditti and Ernst, 1993, Vij and Kaur, 1998, Gangaprasad *et al.*, 2000, Pyati *et al.*, 2002, Decruse *et al.*, 2003, Martin 2007, Janarthanam

and Seshadri, 2008, Medina *et al.*, 2009, Rangsayatorn 2009, Hong *et al.*, 2010, Mata-Rosas *et al.*, 2010, Kaur and Bhutani, 2010, 2013, Rajkarnikar 2011, Pant and Thapa, 2012). *In vitro* multiplication of *V. planifolia* has been reported, through the culture of callus masses (Gu *et al.*, 1987, Davidonis and Knorr, 1991), protocorms, root tips (Philip and Nainar, 1986) and stem-nodes (Kononowicz and Janick, 1984, George and Ravishankar, 1997, Geetha and Shetty, 2000, Kalimuthu *et al.*, 2006, Giridhar and Ravishankar, 2004, Abebe *et al.*, 2009, Palama *et al.*, 2010, Tan *et al.*, 2011). In earlier experiments, MS medium is used to initiate *in vitro* cultures of *V. planifolia*. Presently, attempt has been made to device a simple one-step protocol for propagation of *V. planifolia* using stem-node segments by checking the efficacy of M medium besides MS medium.

Materials and Methods:

Explant and culture medium:

Stem-node segments were excised from *in vitro* grown cultures. Uni-nodal segments (1.5 cm long) were used as explants. These were inoculated on Mitra *et al.*, 1976 (M) medium and Murashige and Skoog 1962 (MS) medium alone and their combinations with growth regulators [6-benzyl aminopurine (BA), 6-furfuryl aminopurine (Kn) and α -naphthalene acetic acid (NAA) at 1.0, 2.0 mg/l] was also evaluated. MS (pH 5.7) and M media (pH 5.8) were supplemented with 3.0 % and 2.0 % sucrose (w/v) (Daurala Sugar Works, India) as a source of nutrition and gelled with 0.8% and 0.9% agar powder (w/v) (Hi-media, Mumbai, India) respectively. In another set of experiment, activated charcoal (AC) 2.0% (w/v) was also used. The pH of MS and M media was adjusted, to 5.7 and 5.8 respectively, with 1 N NaOH/HCl before autoclaving without adding agar. The medium was dispensed in test tubes (25mm \times 150mm) and autoclaved at 121 C at a pressure of 1.06 kg/cm² for 15 min. Autoclaved medium was kept at 37 °C to check for any further contamination.

Inoculations and culture conditions:

All explants, one per test tube were inoculated in an upright position with 5 mm basal portion embedded in the medium. The inoculations were done under aseptic conditions in a laminar airttr flow cabinet. The cultures were incubated at 25 ± 2 °C under 12 hr photoperiod of 3,500 lux light intensity (Flourescent tubes - 40W; Philips India Ltd, Mumbai, India). Eight replicates were used for each experiment and to check the reproducibility the experiment were repeated twice. Sub-culturings were done as and when required.

Observations, experimental design and statistical analysis:

The cultures were observed periodically and data recorded accordingly. All the experiments were conducted using a completely randomized block design. The experiment was repeated

twice. The results presented are the means of at least 8 replications with standard errors. Variance analysis (ANOVA) was performed and comparisons of means were conducted using Tukey Multiple Comparison SPSS 16 version (SPSS Inc., Chicago, US). All analyses were regarded as significant at $P \leq 0.05$.

Results:

Presently, the regeneration response (regeneration percentage, multiplication of *neo*-formations) and was markedly influenced by quality and quantity of PGRs in the nutrient pool. All the explants invariably followed shoot bud mediated pathway of regeneration.

In M medium, regeneration in the explants was obligatory to the use of growth adjuncts in the medium. Addition of BA (2 mg/l) in the medium, favoured 100% regeneration in the nodal discs within 3.78 ± 0.38 week (Table 1). BA (1 mg/l) could initiate regeneration response in 75% explants after 5 week of culture, sprouting the axillary shoot bud. Interestingly, adventive regenerative loci were induced at the nodal region opposite to the axillary bud position (Fig. 1A). The shoot buds grew well into plantlets with 2-3 leaves and 1-2 roots after 16.25 ± 0.19 weeks of culture. BA (2 mg/l) multiplied the single shoot bud by invoking multiple small protuberances developing into shoot buds at its base. A maximum of 6.0 ± 0.81 shoots per explant developed. Mature shoots were sub-cultured in fresh medium. These shoot buds developed into plantlets within 12.80 ± 0.40 weeks of culture. Activated charcoal in the combination favoured early development of plantlets within 11.65 ± 0.41 weeks (Fig. 1D). Replacement of BA with Kn reduced the shoot number/explants, as only 2.25 ± 0.50 shoots per explants were obtained.

In NAA (2 mg/l) supplemented medium, 25 % explants responded to regeneration after 6.70 ± 0.34 weeks of culture. The dormant axillary bud at the node segment invoked a shoot bud which callused at its basal portion (Fig. 1B). The callus was creamish-yellow, compact, hairy all over the surface and organogenetic in nature. Several secondary shoot buds differentiated; these further proliferated and formed shoots (Fig. 1C). The shoot buds were well-defined, green and 0.5 to 1 cm long with round base and pointed tips. These shoot buds developed into plantlets after 17.08 ± 0.09 weeks of inoculations. Additional activated charcoal advanced the development of plantlets; they were formed in 16.20 ± 0.16 of cultures.

In MS medium, the explants responded positively in control (basal) as well as in treatments with cytokinins and auxin. In the basal medium, a single shoot bud sprouted after 6.15 ± 0.19 weeks. 100 % explants regenerated in high concentration of BA (2 mg/l) after 4.13 ± 0.15 weeks of culture (Table 1). The explants followed shoot bud mediated plantlet development in the medium supplemented with BA/Kn (1, 2 mg/l); whereas in NAA (1, 2 mg/l) these shoot buds callused at their basal region after 6 weeks of culture. The callus was yellowish-green, compact, hairy all over the surface and organogenetic. The totipotent callus invoked several growing points that developed into shoot bud. These shoot buds were green in colour and conical in shape. Explants cultured for 8-9 weeks, which had attained the stage of proliferation, consisting of 4-5 shoots, were transferred to fresh medium of same composition. Upon sub-culturing, dwarf shoots grew to normally with more number of axillary shoots. The shoots further elongated and unfolded their leaves and rooted in another 2 weeks developing into plantlets. Additional activated charcoal favoured accelerated development of plantlets. Figure 2A illustrates the pathways followed by shoot bud/s leading to plantlet formation in different nutritional regimes. The axillary bud directly converted into plantlet or underwent multiplication and developed multiple shoot buds and each forming plantlet. Extra adventive shoot buds were invoked at the nodal region, opposite to the axillary shoot bud that formed plantlets. In an

other alternative way the sprouted shoot bud underwent callusing at the base and gave rise to several shootbud growing points which subsequently grew into shoot bud and later into plantlets.

Discussion

The regeneration potential of stem-node explants *in vitro* was positive in *V. planifolia*. Regeneration frequency, number of shoots and their development into plantlets were markedly influenced by chemical stimulus in the nutrient pool. Presently, the efficacy of M medium is tested. Earlier workers mostly used MS medium for initiation and multiplication of *in vitro* cultures using stem-node segments. In the present experiment, the explants regenerated in both the media used i.e. M and MS thereby showing wider nutritional amplitude of the explants. A perusal of literature reveals that, a variety of media is earlier used to induce regeneration response in the stem-node segments of *V. planifolia*. Phillip and Nainar (1986), used four different basal media, viz. Knudson, MS, Gamborg's and SH to culture stem sections and root tips. MS, consisting of high salt concentration, alone was reported to be suitable for optimal plantlet induction and growth. Geetha and Shetty (2000), also used MS medium for initiation of the cultures and N69 basal medium with BA (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. Earlier studies recommend that *in vitro* cultures of *Vanilla*, a basal medium consisting of high salt concentration such as Murashige and Skoog medium is essential at different stages of the culture development (cf. Kalimuthu *et al.*, 2006). From the present study, it appears that a simple defined M medium is also fully capable of initiating the regeneration response in nodal explants.

Moisch *et al.* (1974), has correlated the dormant nature of axillary buds to auxin induced apical dominance in *Dendrobium chrysotoxum* cultures and an anti-auxin treatment helped in breaking down the dormancy in the segments. Similarly trans-cinnamic acid (tCA) was required for plantlet regeneration from *Phalaenopsis* nodal explants (Reisinger *et al.*, 1976). In this experiment, the stem-discs regenerated without the use of any kind of anti-auxin treatment. The response in nodal segments in the basal medium is in accord with earlier results in *Epidendrum O'Brienianum* (Stewart and Button, 1976), *Vanilla planifolia* (George and Ravishankar, 1997), *Renanthera imschootiana* (Laiashram and Sunitibala Devi, 1999).

In this study, cytokinins BA/Kn at 1 mg/l invoked single axillary shoot bud in the nodal region. Higher concentration of BA (2 mg/l) efficiently induced adventive regenerative loci besides the multiplication of axillary buds at their basal portion. A similar response of efficient induction of multiple axillary buds in the nodal explants of *Anoectochilus regalis* and *Anoectochilus sikkimensis* by the use of BA is on records (Gangaprasad *et al.*, 2000). In *Dendrobium macrostachyum* also, high concentration of BA induced multiple axillary shoot formation without intervening callus (Pyati *et al.*, 2002). Similarly the role of cytokinins in induction of multiple shoots has also been reported in *Cymbidium pendulum* (Vij *et al.*, 1994) and *Phalaenopsis* (Duan *et al.*, 1996).

Earlier studies indicated requirement of different nutrient regime for shooting and rooting in the regenerated shoots of *Vanilla planifolia*. Giridhar and Ravishankar (2004), reported differential medium requirement of different stages of development of *Vanilla* cultures; MS medium supplemented with BA and zeatin as multiple shoot induction medium, TDZ with CM 10% for shoot proliferation medium, and N69 fortified with BA and GA₃ for simultaneous shoot multiplication and root initiation. Such a differential requirement at different stages of developing cultures was not observed in this experiment, the shoots successfully rooted in the same medium in which they were formed, without being transferred to any other PGR treatment in M and MS me-

dium.

Presently, a treatment with high concentration of NAA (2 mg l⁻¹) induced callusing at the basal part of axillary bud. Similar role of NAA at high concentration is earlier reported in inducing callus at the cut ends of shoots of *Anoectochilus regalis* (Gan-gaprasad et al., 2000)

Conclusion:

From the present study, it is concluded that quantity and quality of chemical stimulus are important for regeneration and multi-

plication of stem node segments of *Vanilla planifolia*. Besides MS medium, M medium also successfully induced multiple shoots in stem node segments. The shoots rooted in the same medium. Use of suitable chemical stimulus helps in devising an ideal micropropagation system without detriment to the mother plant. Herein, a simple and effective protocol has been reported for *in vitro* propagation of *Vanilla planifolia*.

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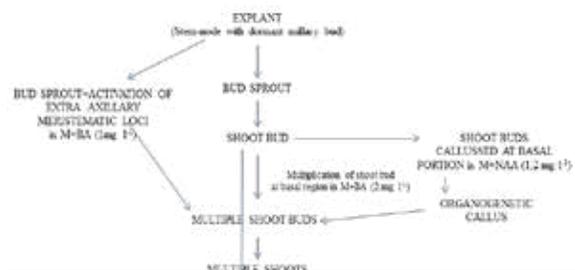
Table 1 *In vitro* regeneration response of stem- node segments of *Vanilla planifolia* on M and MS media with and without growth regulators.

Additives	Regeneration response (%)		Initiation of response (week)		Number of shoots/explant		Complete plantlets (week)	
	M	MS	M	MS	M	MS	M	MS
Basal	0.00±0.00 ^{cd} efghijkl	19.75±0.50 ^{cd} efghijkl	0.00 ^{cd} efghijkl	6.15±0.19 ^{cd} efghij	0.00 ^{ef} ghijkl	1.0±0.00 ^{ef} kl	0.00 ^{cd} efghijkl	17.20±0.16 ^{cd} efghijk
Basal +AC	0.00±0.00 ^{cd} efghijkl	19.75±0.45 ^{cd} efghijkl	0.00 ^{cd} efghijkl	6.20±0.23 ^{cd} efghij	0.00 ^{ef} ghijkl	1.0±0.00 ^{ef} kl	0.00 ^{cd} efghijkl	17.15±0.19 ^{cd} efghijk
BA ₁	70.25±0.50 ^{ab} defghijkl	50.75±0.50 ^{ab} defghijkl	5.25±0.17 ^{ab} defgijk	5.10±0.42 ^{ab} defgijkl	1.25±0.50 ^{ef} kl	1.25±0.5 ^{ef} kl	16.25±0.19 ^g abefghk	12.97±1.00 ^{ab} ghijkl
BA ₁ +AC	71.25±0.50 ^{ab} defghijkl	41.25±0.25 ^{abc} defghijkl	5.25±0.19 ^{ab} cdhijkl	4.50±0.22 ^{ab} defgijkl	1.50±0.57 ^{ef} kl	1.5±0.51 ^{ef} ijkl	16.10±0.20 ^g abefghk	13.35±0.67 ^{ab} hijkl
BA ₂	100.00±0.00 ^{abcd} efghijk	100.00±0.00 ^{abcd} efghijkl	3.78±0.38 ^{abcd} efghij	4.13±0.15 ^{abcd} efghijkl	6.0±0.81 ^{abcd} efghij	5.0±0.81 ^{abcd} efghij	12.80±0.40 ^{abcd} efghijkl	13.62±0.37 ^{ab} hijkl
BA ₂ +AC	100.00±0.00 ^{abcd} efghijk	78.75±0.25 ^{abcd} efghijkl	3.75±0.28 ^{abcd} efghijkl	4.05±0.42 ^{abcd} efghijkl	5.50±1.00 ^{abcd} efghij	6.0±0.86 ^{abcd} efghijkl	11.65±0.41 ^{abcd} efghijkl	12.65±0.41 ^{ab} ghijkl
Kn ₁	50.00±0.00 ^{abcd} efghijk	40.00±0.00 ^{abcd} efghijk	4.38±0.25 ^{abcd} efghijkl	4.38±0.25 ^{abcd} efghijkl	2.25±0.50 ^{ab} efkl	2.25±0.95 ^{ef} ijkl	14.08±0.42 ^g abdefghijkl	14.25±0.17 ^{ab} cdhijkl
Kn ₁ +AC	50.00±0.05 ^{abcd} efghijk	32.50±0.28 ^{abcd} efghijl	4.63±0.47 ^{abcd} efghijkl	4.88±0.25 ^{abcd} efghijkl	2.25±0.95 ^{ab} efkl	1.75±0.50 ^{ef} ijkl	13.70±0.57 ^{ab} cddefghijkl	12.17±0.17 ^{ab} defghijkl
NAA ₁	20.00±0.12 ^{abcd} efghijkl	20.00±0.00 ^{cd} efghijkl	6.70±0.34 ^{abcd} efghijkl	0.00 ^{abcd} efghijkl	2.0±0.81 ^{ab} efkl	0.00 ^{cd} efghijkl	16.30±0.20 ^g abefghk	0.00 ^{abcd} efghijkl
NAA ₁ +AC	20.00±0.87 ^{abcd} efghijkl	20.00±0.00 ^{cd} efghijkl	6.50±0.57 ^{abcd} efghijkl	0.00 ^{abcd} efghijkl	1.50±0.57 ^{ef} kl	0.00 ^{cd} efghijkl	16.40±0.14 ^{ab} efghk	0.0 ^{abcd} efghijkl
NAA ₂	40.00±0.00 ^{abcd} efghijk	28.75±0.50 ^{abcd} efghijk	6.05±0.10 ^{abcd} efgh	6.50±0.40 ^{abcd} efgh	4.50±1.21 ^{abcd} efghij	4.25±0.95 ^{abcd} efghij	17.08±0.09 ^{abcd} efghijkl	16.1±0.08 ^{abcd} efghij
NAA ₂ +AC	38.75±0.25 ^{abcd} efghij	40.00±0.00 ^{abcd} efghijk	5.43±0.43 ^{abcd} efghij	6.05±0.10 ^{abcd} efghij	5.0±0.81 ^{abcd} efghij	4.50±0.57 ^{abcd} efghij	16.20±0.16 ^{ab} efghk	16.32±0.81 ^{cd} efghij

Concentration are subscript = mg l⁻¹. Values in a column with the same superscript are not significantly different at p≤0.05 level.



Fig.1. *In vitro* regeneration response of stem node segments of *Vanilla planifolia* in M and MS medium. (A) Shoot bud developed from extra axillary meristematic loci in BA₁ (1 mg l⁻¹). (B) Callusing of shoot bud at the basal portion in M+NAA₂ (2 mg l⁻¹). (C) Multiple shoots developing in same medium, (D) Plantlets in M+BA₂ (2 mg l⁻¹) + AC enriched medium.



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