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# An Efficient Micropropagation System for *Vitex negundo* L., an Important Woody Aromatic Medicinal Plant, Through Shoot Tip Culture

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**Abstract:** An efficient protocol was established for *in vitro* shoot multiplication from shoot tip explants of *Vitex negundo* on Murashige and Skoog (MS) basal medium supplemented with 8.87 μM 6-benzylaminopurine (BA). Inclusion of 8-Naphthalene Acetic Acid (NAA) in the culture medium along with BA promoted higher rates of shoot multiplication than BA alone. The rate shoot multiplication (6.3) after 4 week of culture on MS basal medium supplemented with 8.87 μM BA, 2.69 μM NAA. The elongated shoots rooted in half strength MS basal salts supplemented with 4.90 μM IBA + 2.85 μm IAA and 2% (w/v) sucrose. The presence of Activated Charcoal (AC) with IBA showed positive response to rooting. *In vitro* propagated plants were transferred to soil with a survival rate of 85% after 1 month.

**Key words:** Nirgundi, verbenaceae, multiple shoots, activated charcoal

## INTRODUCTION

*Vitex negundo* L. (Verbenaceae) is a large woody medicinal plant distributed in some regions of India, Ceylon and China and found to an altitude of 1500 m in the outer Himalayas. This woody shrub is commonly used in different systems of Indian medicine (Anonymous, 1976). All parts of this plant are highly medicinal. The plant is reported to be astringent, cephalic, stomachic, anthelmintic and angiogenic properties (Nadkarni, 1989; Choi *et al*., 2002). Leaves are aromatic and used as a vermifuge. Antifertility activity (Bhargava, 1986) and snake neutralizing activity (Alam and Gomes, 2003) of this plant is reported. The plant is a rich source of two active compounds, betulinic acid and ursolic acid, which are used as antifeedant and antibacterial compounds that protect plants from insect pests (Chandramu *et al*., 2003). In nature, the species propagates through stem cuttings and seeds. Based on our preliminary investigations on propagation with vegetative cuttings are very slow and the survival rate is very limited. Propagation through seed is hindered due to poor germination. Thus, conventional propagation through seeds and vegetative cuttings is not an adequate solution to meet the demand for this rare medicinal plant. Alternative propagation methods would be beneficial in accelerating large-scale multiplication, improvement and conservation of the plant. Tissue culture techniques might be applied to generate large number of true to type propagules. Earlier micropropagation of *V. negundo* by using nodal explants (Sahoo and Chand, 1998; Thiruvengadam and Jayabalan, 2001; Chandramu *et al*., 2003) and through callus cultures (Rani and Nair, 2006) were reported. These reports, however, were inadequate for large-scale propagation of this species and regeneration from callus cultures is an undesirable feature during micropropagation which leads to genetic variability of

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plants (D' Amato, 1975). No more *in vitro* work has been reported by using shoot tip as explant in this plant even though propagation of plants through shoot tip culture allows recovery of genetically stable and true to type progeny (Hu and Wang, 1983).

This research describes successful regeneration of *V. negundo* under *in vitro* conditions using shoot tip explants. We studied the effect of some growth regulators on micropropagation of this species in order to obtain high shoot regeneration rate and high rooting frequency and survival percentage when plantlets were transferred to *ex vitro* conditions.

## MATERIALS AND METHODS

### Plant Material

Shoot tips measuring 1-1.5 cm in length were excised from 2-year-old plants during the month of March from the Botanical Garden of Department of Botany (Kerala), India. These were thoroughly washed with 0.5% Tween-20 solution, disinfected with 70% ethanol for 10 sec and subsequently surface sterilized with $HgCl_2$ solution (0.1% w/v) for 3 min. After rinsing four or five times with sterile distilled water, the explants were implanted vertically on to the culture medium.

### Culture Medium and Culture Conditions

The basal medium used in the present study was that of Murashige and Skoog (1962) supplemented with 3% (w/v) sucrose and different concentrations of 6-benzylaminopurine (BA; 0.0, 2.22, 4.44, 6.66, 8.87, 11.09 and 13.3 μM) or kinetin (0.0, 2.32, 4.65, 6.97, 9.29, 11.6 and 13.9 μM) plus 1-naphthaleneacetic acid (NAA; 1.34 and 2.69 μM) or indole-3-acetic acid (IAA; 1.42 and 2.85 μM) were tested for shoot multiplication. The pH of the media was adjusted to 5.8 using either using 0.1 N NaOH or 0.1 N HCl prior to adding 0.8% (w/v) agar (Qualigens, India). Medium was dispensed in 20 mL aliquots into culture tubes (25×150 mm), which were plugged with non absorbent cotton wrapped in one layer of cheesecloth. Media were steam sterilized at 121°C and 1.05 kg $cm^{-2}$ for 15 min. The cultures were incubated under a 16 h photoperiod in cool white fluorescent light (55 μmol $m^{-2}$ $s^{-1}$). The cultures were maintained by sub culturing at 4 week intervals to fresh medium with the same composition.

### Induction of Rooting and Acclimatization

The elongated shoots (1-2 cm) were excised from the 6 week old culture were used for rooting trials. The excised shoots were transferred to half strength MS basal semisolid medium supplemented with different concentrations and combinations of Indole-3-butyric acid (IBA; 0.0, 0.49, 1.23, 2.46 and 4.90 μM) or IAA (0.0, 0.57, 1.42, 2.85 and 5.71 μM) and 2% sucrose for root initiation. The effect of different concentrations of activated charcoal (AC; 2.0, 3.0 g $L^{-1}$) with IBA (2.46 and 4.90 μM) in the same basal medium was also studied. Culture conditions were the same as for shoot multiplication. The *in vitro* rooted plants were taken out from the medium, washed under tap water to remove all traces of media and then individual plants were transferred to plastic cups containing soil, sand and farmyard manure (1: 1: 1).

### Experimental Design and Data Analysis

All experiments were repeated thrice with 12 replicates each. Standard errors of means were calculated and statistically significant mean differences were determined by the Least Significant Difference (LSD) test.

## RESULTS AND DISCUSSION

### Effect of Growth Regulators on Shoot Multiplication

Shoot tips were induced on MS medium supplemented with varying concentrations and combinations of cytokinins and auxins to evaluate the effect of growth regulators on *V. negundo* shoot

Table 1: Influence of plant growth regulators on morphogenic response from shoot tip explants of *Vitex negundo* on MS medium after 4 week of culture

| Growth regulators (µM) | Shoot development (%) | Shoot number/explant | Shoot length |
|---|---|---|---|
| BA | | | |
| 0.0 | 0 | 0 | 0 |
| 2.22 | 65±0.7$^{d}$ | 1.2±0.2$^{b}$ | 1.6±0.7$^{bc}$ |
| 4.44 | 72±0.8$^{e}$ | 2.1±0.8$^{c}$ | 2.2±1.2$^{c}$ |
| 6.66 | 75±0.2$^{f}$ | 3.4±1.2$^{f}$ | 2.8±1.6$^{d}$ |
| 8.87 | 76±0.5$^{f}$ | 4.2±1.3$^{fg}$ | 1.8±0.8$^{c}$ |
| 11.09 | 73±0.6$^{e}$ | 2.8±0.6$^{e}$ | 1.2±0.2$^{b}$ |
| 13.3 | 69±1.2$^{e}$ | 1.6±0.9$^{bc}$ | 0.9±0.4$^{ab}$ |
| Kinetin | | | |
| 0.0 | - | - | - |
| 2.32 | 32±1.2$^{a}$ | 0.2±1.6$^{a}$ | 0.2±1.6$^{a}$ |
| 4.65 | 43±1.4$^{b}$ | 0.7±1.2$^{a}$ | 0.6±1.2$^{a}$ |
| 6.97 | 56±0.5$^{c}$ | 0.9±0.3$^{ab}$ | 2.1±0.9$^{c}$ |
| 9.29 | 44±0.7$^{b}$ | 1.1±0.9$^{b}$ | 1.6±1.1$^{bc}$ |
| 11.6 | 42±1.3$^{b}$ | 0.9±1.1$^{ab}$ | 1.4±0.7$^{b}$ |
| 13.9 | 47±0.7$^{bc}$ | 1.2±0.7$^{b}$ | 1.3±0.2$^{b}$ |
| BA+kinetin | | | |
| 2.22+2.32 | 54±1.4$^{c}$ | 2.0±0.9$^{c}$ | 1.6±1.1$^{bc}$ |
| 4.44+2.32 | 64±0.6$^{e}$ | 2.8±1.6$^{e}$ | 1.5±0.4$^{b}$ |
| 2.22+4.65 | 61±0.8$^{e}$ | 1.2±0.8$^{b}$ | 1.2±0.2$^{b}$ |
| 4.44+4.65 | 52±0.2$^{d}$ | 2.6±0.4$^{de}$ | 1.3±1.1$^{b}$ |
| BA+NAA | | | |
| 4.44+1.34 | 66±1.1$^{de}$ | 3.8±0.2$^{f}$ | 1.5±0.2$^{b}$ |
| 8.87+1.34 | 72±0.8$^{e}$ | 4.1±1.6$^{fg}$ | 2.2±0.6$^{c}$ |
| 4.44+2.69 | 70±0.9$^{e}$ | 3.7±0.6$^{f}$ | 2.6±0.8$^{d}$ |
| 8.87+2.69 | 62±0.7$^{e}$ | 6.3±0.4$^{i}$ | 2.7±1.3$^{d}$ |
| BA+IAA | | | |
| 4.44+1.42 | 55±0.4$^{c}$ | 1.1±0.2$^{b}$ | 0.8±0.8$^{ab}$ |
| 8.87+1.42 | 62±0.2$^{d}$ | 1.8±1.0$^{c}$ | 1.1±0.7$^{b}$ |
| 4.44+2.85 | 64±0.9$^{d}$ | 1.3±0.4$^{b}$ | 1.8±0.6$^{c}$ |
| 8.87+2.85 | 58±0.6$^{cd}$ | 1.9±0.6$^{c}$ | 1.7±0.8$^{c}$ |

Treatment means followed by same superscripts within column are not significantly different from each other ($p<0.05$); comparison by LSD Multiple Range test

multiplication (Table 1). Shoots were responded within 12-16 days of inoculation (Fig. 1A). There was no sign of growth when shoot explants were cultured in media without cytokinin or auxin. At higher concentrations of BA or kinetin, the response in terms of shoot growth and multiplication was not favourable. An average number of 4.2 shoots/explant was observed on MS medium having 8.87 µm BA and 3% sucrose within 4 week of culture (Fig.1B). Comparing BA and kinetin, the former was found to be more effective than later. This is in concordance with the results of Sahoo and Chand (1998) and Chandramu *et al*. (2003) and agreement with the findings of Rani and Nair (2006) in their callus regeneration studies. The combination of BA and kinetin showed good response of multiplication and elongation. Cytokinins were shown to be the most critical growth regulators for shoot proliferation of many medicinal plants (Chen *et al*., 1995; Rout *et al*., 2000; Martin *et al*., 2005; Raghu *et al*., 2006). Inclusion of either NAA or IAA in the culture medium along with BA favored shoot multiplication and elongation. Shoot multiplication was enhanced when BA containing medium was supplemented with NAA compared with IAA. Many authors reported that cytokinin was required in optimal quantity for shoot proliferation in *Mentha arvensis* (Shasany *et al*., 1998), *Pinellia ternate* (Tsay *et al*., 1989) and *Gentiana kurroe* (Sharma *et al*., 1993) but inclusion of a low concentrations of auxin along with cytokinin increased the rate of shoot multiplication (Rout *et al*., 2000). The maximum number (an average of 6.3) of shoots/ explant was observed on medium having 8.87 µM BA, 2.69 µM NAA and 3% sucrose within 4 week of culture (Fig. 1C). The rate of multiplication was not declined as the number of subcultures increased. Every subculture was made at 4 week intervals. Raghu (2006) observed similar results in tree species like *Aegle marmelos* and *Oroxylum indicum*.


Fig. 1: Micropropagation of *Vitex negundo* by using shoot tip explants. A: Shoot induction on MS medium supplemented with cytokinins after 12-16 days of culture. B: Shoot multiplication on MS medium supplemented with 8.87 µM BA, 2.69 µM 3 NAA and 3% source (w/v) after 4 week of culture. C: Shoot multiplication on MS medium supplementws with 8.87 µM BA, 2.69 µM NAA and 3% sucrose (w/v) after 4 week of culture. D: Rooting of *in vitro* derived hoots on half strength MS medium containing 4.90 µM IBA + 2.85 µM IAA with 2% source after 16 days of culture. E : Rooting of *in vitro* derived shoots on half strength MS medium containing 4.90 µM IBA+ 3 g $L^{-1}$ AC with 2% source after 10 days of culture. F, Acclimatized plantlet with flower buds

Table 2: Effect of auxins and Activated Charcoal (AC) on rooting response of *Vitex negundo* on half strength MS medium after 3 week of culture

| IBA (μM) | IAA (μM) | AC (g $L^{-1}$) | (%) of shoots rooted | No. of roots per shoot | Days to rooting |
|---|---|---|---|---|---|
| 0 | 0 | | 0 | 0 | 0 |
| 0.49 | | | 41.2±0.7$^{e}$ | 1.2±0.8$^{a}$ | 14-18 |
| 1.23 | | | 58.3±0.6$^{d}$ | 1.7±1.6$^{ab}$ | 12-16 |
| 2.46 | | | 78.1±0.9$^{g}$ | 2.7±1.3$^{c}$ | 12-16 |
| 4.90 | | | 81.0±1.8$^{g}$ | 3.5±0.3$^{d}$ | 10-14 |
| | 0.57 | | 12.1±0.2$^{a}$ | 1.1±1.7$^{a}$ | 14-18 |
| | 1.42 | | 18.3±0.4$^{ab}$ | 1.2±1.2$^{a}$ | 14-18 |
| | 2.85 | | 19.7±0.6$^{ab}$ | 1.4±1.3$^{a}$ | 14-18 |
| | 5.71 | | 20.4±1.2$^{b}$ | 1.7±0.6$^{ab}$ | 12-16 |
| 4.90 | 2.85 | | 94.1±0.6$^{f}$ | 3.8±0.2$^{d}$ | 10-14 |
| 2.46 | 5.71 | | 79.6±0.1$^{e}$ | 2.9±0.1$^{c}$ | 12-16 |
| 2.46 | | 2.0 | 78.8±0.2$^{e}$ | 2.4±0.4$^{c}$ | 10-14 |
| 2.46 | | 3.0 | 81.6±1.1$^{e}$ | 2.9±0.3$^{c}$ | 8-12 |
| 4.90 | | 2.0 | 88.5±1.2$^{ef}$ | 3.1±1.1$^{cd}$ | 8-12 |
| 4.90 | | 3.0 | 92.6±1.3$^{f}$ | 3.4±0.8$^{d}$ | 8-12 |

Treatment means followed by same superscripts within column are not significantly different from each other ($p<0.05$); comparison by LSD multiple range test

**Rooting of Microshoots**

Elongated shoots (1-2 cm) were excised from 6 week old cultures were transferred to half strength MS basal semisolid medium supplemented with different concentrations and combinations of Indole-3-butyric acid (IBA; 0.0, 0.49, 1.23, 2.46 and 4.90 μM) or IAA (0.0, 0.57, 1.42, 2.85 and 5.71 μM) and 2% sucrose for root initiation. The effect of activated charcoal (2.0, 3.0 g$L^{-1}$) with IBA (2.46 and 4.90 μM) in the same basal medium was tried for root induction (Table 2). MS medium without growth regulators did not promote shoot induction. The percentage of shoots that formed roots and the number of roots per shoot varied significantly with different concentrations of IBA, IAA, or IBA+ IAA. Optimal rooting (94.1%) with no intervening callus was observed within 10-14 days of transfer to medium containing 4.90 μM IBA+ 2.85 μM IAA with 2% sucrose (Fig. 1D). Root development was, however slow at higher concentrations of auxins used (data not shown). Inclusion of AC with IBA favored good root induction in earlier days (Table 2; Fig. 1E). The positive response of rooting by AC in the present study is similar to observations on *Chlorophytum borivilianum* (Purohit *et al*., 1994) and *Clerodendrum colebrookianum* (Mao *et al*., 1995). It can be attributed to the capability of this compound to adsorb the impurities in the culture medium (Weatherhead *et al*., 1979) and modification of the availability of the nutrients (Ebert *et al*., 1993). Rooted plants were transferred to plastic cups containing soil, sand and farmyard manure (1: 1: 1). About 85% plants were surviving one month after transfer. The acclimatized plants exhibited normal growth true-to-type morphology, with flowering (Fig. 1F).

In conclusion, we report an efficient and easy to handle protocol for successful micropropagation of an important medicinal plant, *Vitex negundo*. This protocol provides a successful and rapid technique that can be used for ex-situ conservation.

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