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High Frequency Induction of Multiple Shoots from Nodal Explants of *Vitex negundo* L. Using Sodium Sulphate

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Abstract

The effect of sodium sulphate on shoot induction and multiple shoot formation from nodal explants of *Vitex negundo* L. was tested on Murashige and Skoog's (MS) medium fortified with different auxins, cytokinins and sucrose. Highest percentage (97.78%) of explants for shoot induction and multiple shoot (20.68/explant) production were observed in the combination treatment of N^6-Benzyl adenine (BA) (17.80 μM/L), α-Naphthalene acetic acid (NAA) (2.15 μM/L) and 5% sucrose supplemented with 100 mg/L sodium sulphate. *In vitro* raised shoots were rooted on the half-strength MS medium fortified with different concentrations of NAA, Indole-3-acetic acid (IAA), and Indole-3-butyric acid (IBA) alone and in combinations. Among the treatments, 4.90 μM/L of IBA was found most effective (95.56%) in inducing roots. The rooted plantlets were shifted to glasshouse for acclimatization and later transferred to the field with cent percent survival. Furthermore, *in vitro* flowering was observed in the shoots cultured on MS medium supplemented with BA (8.90 μM/L) and NAA (1.61 μM/L).

Key words: Medicinal plant, multiple shoots, nodal explants, sodium sulphate, *Vitex negundo*, sucrose

Introduction

Vitex negundo L., a member of verbenaceae, is an important medicinal shrub found throughout the Indian subcontinent. The extract of leaves is used for caterrhal fever and vermifuge (Chadha 1976; Hussain et al. 1992) and used extensively in Ayurvedic and Unani medicine (Kapur et al. 1994). The plant was found to possess anti-arthritic (Tamhanker and Saraf 1994), hepatoprotective (Kapur et al. 1994), anti-inflammatory, anti-allergic (Chawla et al. 1991), insecticidal (Aswal et al. 1996), antibacterial (George et al. 1947), anti-fungal (Rusia and Srivastava 1988) as well as mosquito repellant activities (Hebbalkar et al. 1992). The *Vitex* leaf extract revealed anticancer activity against *Echrlich ascites* tumor cells (Ambasta 1986). Recently, betulinic acid, ursolic acid and β-sitosterol have been isolated from the leaves of *Vitex negundo* (Chandramu et al. 2002). Betulinic and ursolic acids were found to possess anticancer (Noda et al. 1997) and anti-HIV (Fujioka et al. 1994; Xu et al. 1996) properties; while β-sitosterol showed angiogenic properties (Choi et al. 2002).

Unscrupulous and unscientific management practices have threatened the existence of medicinal plant species, which are consequently facing extinction. Besides, there is an ever increasing demand for phytoceuticals because of their short supply. Intensive studies on medicinal plant species pertaining to their conservation, cultivation, selection and genetic enrichment of germplasm are desperately needed.

Propagation of *Vitex negundo* L., through seeds is hindered due to poor germination. Conventional propagation with vegetative cuttings is very slow and a large number of cuttings do not survive during transport and plantation (Sahoo and Chand 1998). *In vitro* techniques paved the way for mass production of plants in a short time span to meet the increasing demand. In recent years, tissue culture techniques are extensively used for the propagation of endangered medicinal plants (Pattnaik and Chand 1996; Thoyajaksha and Rai 2001). However, very few reports exist on the micropropagation of *Vitex negundo* (Sahoo and Chand 1998; Thiruvengadam and Jayabalan 2000; 2001).

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The present study was undertaken to optimize a protocol for high frequency induction of multiple shoots from the nodal explants and regenerate plants of *Vitex negundo* to meet its demand in medicine and agriculture.

Materials and Methods

Plant materials

Actively growing and healthy shoot material of *Vitex negundo* L., with three to four nodes, were collected from an adult plant growing in the Osmania University Campus (Hyderabad), India. After removing the leaves, the shoots were cut into pieces (0.5-1.0 cm), each containing a single node with dormant axillary buds. These explants were thoroughly washed under running tap water for 30 min and were treated with 5% (vol/vol) laboline (Qualigens, India) for 7-8 min followed by distilled water. The shoots were surface-sterilized with 0.05% (wt/vol) $HgCl_2$ for 20 min and rinsed with sterile distilled water for five to six times.

Culture media and culture conditions

Murashige and Skoog's (MS) medium (1962) containing 100 mg/L (wt/vol) sodium sulphate, 5% (wt/vol) sucrose, fortified with Cytokinins [N^6- Benzyladenine (BA) (4.40-22.20 μM/L) or Kinetin (KN) (4.60-23.20 μM/L)] and Auxins [Naphthalene acetic acid (NAA) (0.54-2.67 μM/L), Indole-3-butyric acid (IBA) (0.49-2.46 μM/L) or Indole-3-acetic acid (IAA) (0.57-2.85 μM/L)], either individually or in combinations, was used. The pH of the medium was adjusted to 5.7-5.8 before adding 0.9% agar-agar (Hi-Media, India). Molten medium (20 mL) was poured into test tubes (2.5×12 cm; Borosil, India) and in 250mL Erlenmeyer Flasks (Borosil, India), and was autoclaved at 15 lb and 121C for 15 min. All the cultures were incubated at 252C at a relative humidity of 60-65% under 16h photoperiod of 35-50 μmole $m^{-2}s^{-1}$ irradiance provided by cool-white fluorescent tubes (Crompton Greaves, India).

Shoot induction

To study the effect of different concentrations of sucrose and sodium sulphate on the production of multiple shoots, the surface-sterilized nodal explants were cultured on MS medium supplemented with BA (17.80 μM/L), NAA (2.15 μM/L), sucrose (2-8%) and sodium sulphate (0-200 mg/L). After 20 days of inoculation, the explants were transferred to fresh medium. After 6 weeks of culture, data were recorded on shoot induction and the number of shoots per explant.

Multiplication of cultures

In vitro raised shoots were cut into pieces containing a single node along with dormant axillary buds and were cultured on MS medium supplemented with sodium sulphate (100 mg/L), BA (17.80 μM/L) and NAA (2.15 μM/L) for the induction of multiple shoots. Subsequently, subcultures were done at 25-day interval to study the effect of culture passages on the explant response for shoot induction and multiple shoot formation.

Rooting of shoots

After six weeks, 5-6 cm long shoots, having 6-8 compound leaves, were rooted on half-strength MS medium containing 2% (wt/vol) sucrose and 0.8% (wt/vol) agar-agar. The medium was supplemented with varied concentrations of auxins: NAA (2.67-16.07 μM/L), IAA (2.85-17.13 μM/L), and IBA (2.46-14.70 μM/L).

Acclimatization and transfer of plantlets to field

Plantlets with well developed roots were gently washed under running tap water, rinsed with sterile-distilled water and transferred to pots containing either autoclaved garden soil, or a mixture of red soil, clay and sand in the ratio of 3:2:1, or soilrite mix (Keltech Energies Ltd., India) or vermicompost (Local made). For further growth, the potted plants were sprinkled with Hoaglands solution (Hoaglands and Arnon 1950) for 3 weeks at 3-day interval; later the plants were watered for 3 weeks, and were maintained in the glass house at 25±3°C and 80-85% relative humidity. Subsequently, these plants were transferred to the experimental field for further growth and development.

Statistical analysis

All experiments were repeated thrice each consisting of 15 replicates. The data were was analyzed using analysis of variance (ANOVA) with Matlab software version 5.3. Percentage values were subjected to angular transformations (arcsine values) because of binomial proportion (Snedecor and Cochran 1968).

Results and Discussion

Initiation and proliferation of nodal cultures

Preliminary experiments were conducted for selection of explant to produce more number of shoots. The nodal explant

was found to be more effective for *in vitro* propagation of *Vitex negundo* L., cultured on the MS medium supplemented with various phytohormones, when compared to other explants, viz., shoot tips, internodes and leaves.

The nodal explants cultured on MS medium, supplemented with various concentrations of BA or KN individually or in combination with NAA, IAA or IBA have developed healthy shoots. When nodal explants were cultured on MS media fortified with cytokinins alone also induced adventitious shoots at a lesser frequency compared to the media supplemented with combination treatments of cytokinin and auxin (Table 1). Between the two cytokinins tested, BA was found to be more effective than KN in the induction of multiple shoots from the nodal explants. Similar observations were reported in other medicinal and aromatic plant species: *Withania somnifera* (Sen and Sharma 1991), *Ocimum* spp. (Pattnaik and Chand 1996) and *Dictyospermum ovalifolium* Wight (Thoyajaksha and Rai 2001). The bud breaking and shoot induction in cultures of nodal explants indicate the function of cytokinins (Sahoo and Chand 1998). In the present investigation, bud breaking and multiple shoot induction was increased in treatments of BA up to 17.80 μM/L (4.0 mg/L) when supplemented with sodium sulphate (100 mg/L). Sahoo and Chand (1998) reported a similar increase in the percentage of bud breaking and multiple shoot induction with increasing BA concentration up to 8.90 μM/L (2.0 mg/L) in *Vitex negundo*; however a declining trend was observed beyond this dosage. This increase in the bud breaking and multiple shoot induction in the present study may be attributed to the synergistic effect of sodium sulphate and BA. A reduction in the number of shoots per explant was observed when the BA level increased beyond the optimal concentration (17.80 μM/L). These results agree with the earlier studies on medicinal plants (Sen and Sharma 1991; Vincent et al. 1992) and woody plants (Lakshmanan et al. 1997; Pattnaik and Chand 1997). The percentage of explants responding for shoot induction (90-96%) and multiple shoot formation/explant (4-20) increased significantly on medium containing sodium sulphate (100 mg/L) along with optimum levels of BA (17.80 μM/L) and NAA (0.54-2.67 μM/L). The BA and NAA along with sodium sulphate exhibited a synergistic effect on the percentage response of explants for shoot induction and multiple shoot formation. In each explant, 5-8 axillary buds were formed within 10-15 days after inoculation (Figure 1A). Later, 25-30 days after inoculation, new shoots (10-25) were developed adjacent to these axillary buds (Figure 1B). The number of shoots per explant increased when the media were replaced afresh on every twentieth day of inoculation. The percentage of explants for shoot induction and number of shoots per explant increased with increasing concentration of NAA up to 2.15 μM/L. Thiruvengadam and Jayabalan (2000) also found similar effects in *Vitex negundo*, when nodal explants were cultured on the medium containing BA and NAA. Studies of Mercier et al. (1992) in *Gomphrena officinalis* and Mathur et al. (1987) in *Rauvlfia*

Table 1. The effect of different concentrations of cytokinins, individually and in combination with auxins, on shoots induction and multiple shoot formation of nodal explants of *Vitex negundo* L.

Cytokinin/ Auxin	Concentration (μM/L)	% of explant response for Shoot induction (Mean±SE)	No. of Shoots/ explant (Mean±SE)	Shoot length (cm) (Mean±SE)
Basal	-	-	-	-
KN	4.60	37.78±2.72	1.35±0.15	1.56±0.17
	9.30	48.89±2.71	1.95±0.21	1.77±0.10
	13.90	60.00±4.72	2.11±0.24	2.10±0.17
	18.60	57.78±2.72	2.04±0.20	2.34±0.14
	23.20	51.11±2.22	1.69±0.19	1.79±0.11
BA	4.40	62.22±3.33	1.96±0.16	2.85±0.17
	8.90	73.33±4.71	2.73±0.23	3.22±0.14
	13.30	84.44±3.33	3.95±0.22	3.43±0.12
	17.80	86.67±4.72	6.26±0.30	4.21±0.13
	22.20	80.00±4.71	1.94±0.14	2.39±0.09
KN+NAA	18.60+0.54	57.78±2.72	2.35±0.21	2.54±0.13
	18.60+1.07	62.22±3.85	2.79±0.24	2.63±0.15
	18.60+1.61	73.33±4.71	3.18±0.25	2.79±0.17
	18.60+2.15	68.89±2.72	3.03±0.25	3.19±0.14
	18.60+2.67	60.00±7.19	2.96±0.23	2.12±0.13
KN+IAA	18.60+0.57	57.76±2.48	2.08±0.17	2.39±0.14
	18.60+1.14	62.22±2.72	2.54±0.16	2.91±0.13
	18.60+1.71	71.11±2.71	2.97±0.17	3.02±0.12
	18.60+2.28	75.56±2.72	2.47±0.17	2.44±0.11
	18.60+2.87	60.00±0.00	2.30±0.19	1.96±0.07
KN+IBA	18.60+0.49	55.56±3.33	2.00±0.16	2.41±0.14
	18.60+0.98	60.00±4.71	2.67±0.14	2.55±0.12
	18.60+1.48	68.89±1.56	3.06±0.17	2.80±0.13
	18.60+1.97	64.44±3.14	2.76±0.15	3.09±0.08
	18.60+2.46	57.76±2.48	2.04±0.22	2.64±0.14
BA+NAA	17.80+0.54	88.89±2.71	4.43±0.26	3.17±0.14
	17.80+1.07	91.11±2.71	8.63±0.48	5.20±0.17
	17.80+1.61	95.56±2.72	11.30±0.45	5.03±0.15
	17.80+2.15	97.78±2.72	20.68±0.56	4.54±0.14
	17.80+2.67	80.00±0.00	6.47±0.53	3.04±0.15
BA+IAA	17.80+0.57	68.89±2.71	4.19±0.26	2.70±0.16
	17.80+1.14	75.56±2.72	5.97±0.34	2.80±0.17
	17.80+1.71	88.89±2.71	5.13±0.21	3.53±0.13
	17.80+2.28	82.22±2.72	4.54±0.19	2.36±0.10
	17.80+2.87	73.33±4.71	3.48±0.23	2.34±0.13
BA+IBA	17.80+0.49	64.44±2.72	3.76±0.25	2.59±0.18
	17.80+0.98	86.67±0.00	4.00±0.25	2.70±0.16
	17.80+1.48	91.11±2.71	3.29±0.14	2.71±0.12
	17.80+1.97	82.22±2.72	3.19±0.14	3.01±0.16
	17.80+2.46	71.11±2.71	3.06±0.15	2.58±0.03

serpentina also revealed the enhancing effect of medium fortified with NAA and BA in shoot multiplication.

In vitro flowering was observed in plantlets after 30 days of inoculation on the MS medium supplemented with BA (8.90 μM/L) and NAA (1.61 μM/L) (Figure 1C). This observation is in agreement with the results of Thiruvengadam and Jayabalan (2001) in *Vitex negundo*. *In vitro* flowering was attributed to the effect of culture conditions besides genetic factors (Tisseral and Galletta 1993).

Effect of sodium sulphate and sucrose on the induction of multiple shoots

To study the effect of sodium sulphate, in the presence of sucrose, on the induction of multiple shoots, the nodal explants were cultured on a medium supplemented with sodium sulphate (0-200 mg/L), sucrose (2-8%) along with optimal concentrations of BA (17.80 μM/L) and NAA (2.15 μM/L). The conc. of 100 mg/L of sodium sulphate was found most effective in the induction of multiple shoots, compared to other concentrations (Figure 2). Chandler and Thorpe (1987), observed an increase in the accumulation of proline and decline in the water potential in the callus cultures of *Brassica napus* grown on sodium sulphate. Proline accumulation is the widespread phenomenon observed in the plant cells when exposed to salt or water stress (Chandler et al. 1986). Callus cultures of plants were found to accumulate proline and ABA under salt and water stress conditions (Kishor et al. 1999). Exogeneously supplied proline stimulated the cytokinin-mediated shoot formation in *Cucumis* (Shetty et al. 1992) and auxin induced embryogenesis in *Medicago* (Shetty and Mc Kersic 1993). A mitochandrial enzyme, proline dehydrogenase, might play an important role during organogenesis (Kishor et al. 1999). Chandler et al. (1986) observed a decline in the proline accumulation in subsequent culture passages on the medium containing sodium sulphate, and noticed a negative correlation between proline accumulation and callus growth.

Sodium sulphate, at the optimum concentration (100 mg/L) with 5% sucrose was found most effective in the induction of multiple shoot from the nodal explants, and however, an increase and decrease in the sucrose levels reduced the number of shoots/explant (Figure 2). Increase in the induction of multiple shoots (20 shoots/explant) may be due to the synergistic effect of sucrose and sodium sulphate. Boggetti et al. (1999) observed a gradual increase in shoot multiplication and shoot elongation from the nodal explants of cashew nut (*Anacardium occidentale*) upto 4% sucrose and then there was a gradual decline.

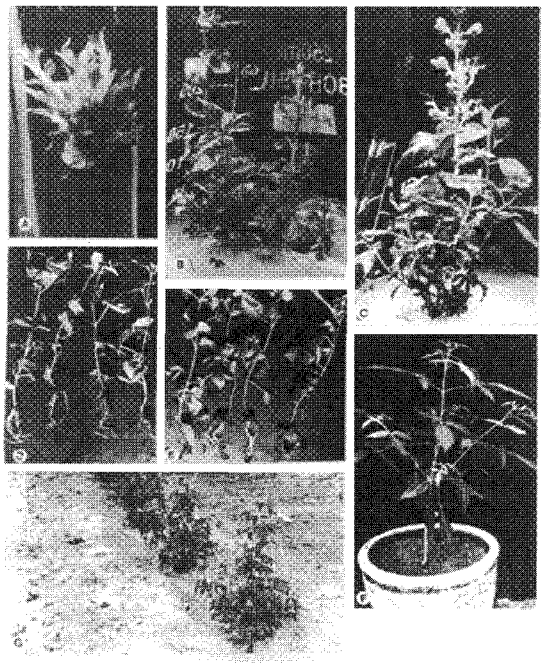

Figure 1. Micropropagation of *Vitex negundo* L.; A) 15-day old multiple shoots, B) 35-day-old multiple shoots, C) *In vitro* flowering, D) Rooting on half-strength medium supplemented with IAA and IBA, E) Rooting on half-strength medium supplemented with IBA, F) Acclimatization of plantlets in glass house and G) plants established in the field.

Multiplication of shoots

The effect of culture passages was studied on multiple shoot

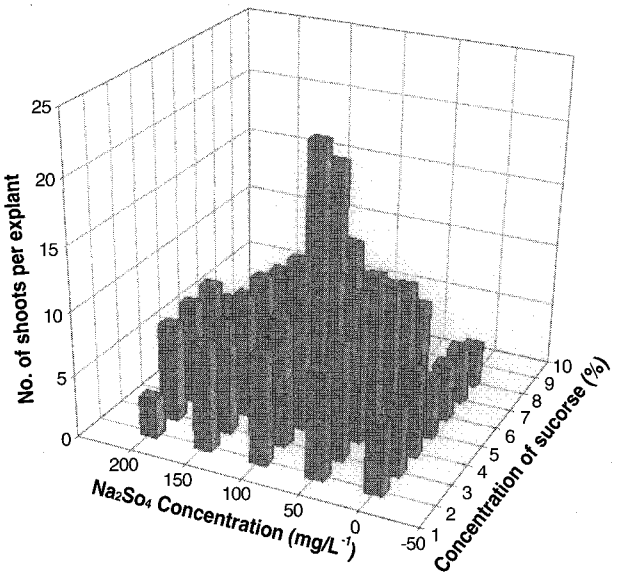

Figure 2. The effect of sodium sulphate, in the presence of sucrose, on multiple shoot induction from nodal explants of *Vitex negundo* L. on MS media supplemented with BA and NAA.

induction in the subcultures of nodal segments of *in vitro* raised shoots (25 day-old) on MS medium supplemented with BA (17.80 μM/L) and NAA (2.15 μM/L) along with sodium sulphate (100 mg/L). The highest response of nodal explants (98-100%) with a maximum average number of shoots (20) per explant was observed in the first five culture passages and then there was a gradual decline (Figure 3 A and B). In *Vitex negundo*, similar observations were made by Sahoo and Chand (1998) when subcultured on MS medium supplemented with BA [(4.40 μM/L) (1.0 mg/L)] and GA_3 [(1.15 μM/L) (0.4 mg/L)] upto 2 subcultures and then there was a gradual decline. A decline in the proline accumulation was observed in the subsequent culture passages of *Brassica napus* on the medium containing sodium sulphate, and higher concentrations of sodium sulphate increased the necrosis (Chandler and Thorpe 1987). In the present investigation, a gradual decline in the number of shoots from the sixth culture passage onwards and a complete necrosis of shoots was observed at the ninth culture passage. This may be due to decrease in the proline accumulation in the cultures.

The addition of sodium sulphate did not cause much difference in the percentage of explant response for shoot induction (Figure 3A); however, there was a 2-3 fold increase in the multiple shoot production (Figure 3B). The results observed in culture passages, without sodium sulphate, are in agreement with the observations of Sahoo and chand (1998).

Rooting of shoots

Shoots regenerated from nodal explants failed to produce roots on half strength MS basal medium (Table 2). Whereas, the medium supplemented with NAA induced callus at the base of the shoots without any roots. However, lower concentration of NAA (2.67 μM/L) induced one or two roots along with calli. Whereas, IAA contained medium induced roots (2 to 4 roots/shoot) in 38% of cultures. On the other hand, the individ-

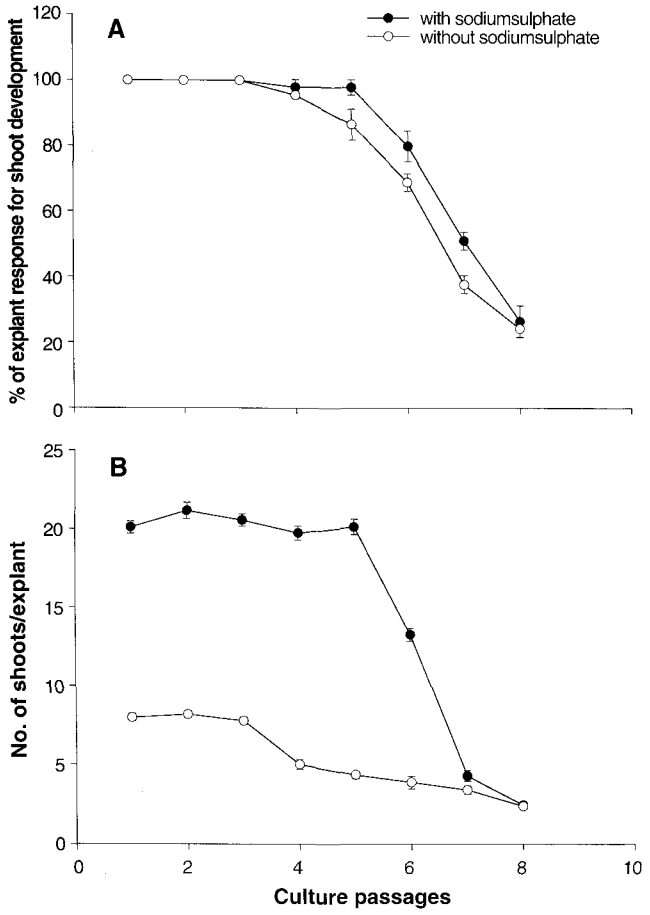

Figure 3. The effect of culture passages on A) Percentage of explant response for shoot induction B) Multiple shoot formation.

Table 2. The effect of auxins, individually and in combination, on rooting of *in vitro* raised shoots of *Vitex negundo* L.

Auxin	Concentration (s) (μM/L)	% of rooting (Mean±SE)	No. of roots/shoot (Mean±SE)	Root length (cm) (Mean±SE)
Basal	-	-	-	-
NAA	2.67	(8.89±4.58)*	1.25±0.40	0.75±0.17
	5.39	**	-	-
	10.68	***	-	-
	16.07	***	-	-
IAA	2.85	11.11±2.71	1.80±0.27	2.32±0.31
	5.71	22.22±3.33	3.00±0.22	3.05±0.29
	11.42	42.22±2.72	3.33±0.17	3.76±0.21
	17.13	31.11±2.71	3.07±0.29	3.57±0.26
IBA	2.46	46.67±4.71	5.76±0.29	4.38±0.19
	4.90	95.56±1.56	8.95±0.32	5.26±0.09
	9.80	80.00±2.72	5.81±1.39	4.81±0.08
	14.70	51.11±2.71	5.65±0.37	4.42±0.14
IAA+NAA	2.85+2.67	**	-	-
	2.85+5.39	*	-	-
	5.71+2.67	37.78±2.48*	2.24±0.37	2.22±0.16
	5.71+5.39	17.78±3.33*	2.63±0.21	1.83±0.29
IBA+NAA	2.46+2.67	08.89±2.71*	1.25±0.66	1.65±0.63
	2.46+5.39	*	-	-
	4.90+2.67	22.22±2.72 *	3.00±0.90	2.94±0.37
	4.90+5.39	62.22±2.72 *	3.07±0.15	2.13±0.15
IAA+IBA	2.85+2.46	44.44±2.48	5.40±0.33	4.24±0.12
	2.85+4.90	88.89±2.71	5.70±0.28	4.62±0.12
	5.71+2.46	86.67±4.71	5.67±0.28	4.51±0.15
	5.71+4.90	91.11±2.72	5.83±0.34	4.62±0.14

- = No response; * = formation and size of callus
* = < 1.0 cm; ** = 1-2 cm; *** = > 2 cm

ual treatment of IBA showed the highest rooting (Table 2). The auxin, NAA in combinations with IAA or IBA induced callus from the base of the shoot. About 90% of shoots have developed the roots (8-12 roots/shoot) on medium fortified with IBA (2.46-4.90 μM/L) and IAA (2.85-5.71 μM/L). Similar observations were noticed in sweetgum (Kim et al. 1997). Among the auxins employed, IBA was found to be the most effective in the induction of roots without inducing callus (Table 2). Thiruvengadam and Jayabalan (2000) also made similar observations in *Vitex negundo* at similar levels of IBA. Whereas, the optimal concentration of IBA (4.90 μM/L) induced roots from 96% of the shoots. These roots were thick with secondary roothairs, which help in establishing the plantlets in the soil (Figure 1E). However, the roots induced on the medium containing IAA alone and in combination with IBA were too slender (Figure 1D). These results are in agreement with the observations made by Swamy et al. (1992) in rose wood.

Acclimatization and transfer of plantlets to field

Well-developed, *in vitro* rooted plants were transferred to four types of planting material in the glass house for acclimatization (Table 3) (Figure 1F). Among these, the vermicompost proved to be the most suitable for hardening of *Vitex negundo* plants with a maximum survival rate of 96% followed by 93% in soilrite mix (Table 3). After hardening in the glass house the plants were transferred to the field with cent percent survival rate (Figure 1G). These plants did not show any detectable variation in the morphology or growth characteristics when compared to the control plants.

This is the first report of its kind dealing with the establishment of *in vitro* raised plants in the field, and the protocol optimized is highly useful for mass multiplication of *Vitex negundo* in a short time for the production of secondary metabolites.

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Table 3. The effect of different substrates on acclimatization and hardening of *in vitro* raised plants in glasshouse conditions.

Substrate	No. of plantlets	No. of plants survived	% of survival	Shoot length (cm) (Mean±SE)	No. of leaves/plant (Mean±SE)
Garden soil	24	15	62.50	25.53±2.15	22.53±1.57
Vermicompost	27	26	96.29	54.65±1.59	46.50±1.56
Mixture of soils	30	21	70.00	32.76±2.55	32.09±1.17
Soilrite mix	30	28	93.33	42.53±1.62	38.03±1.38

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