

An efficient protocol for *in-vitro* regeneration of *Vitex negundo* an important medicinal plant

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ABSTRACT

An efficient *in vitro* protocol has been established for propagation of elite plant of *Vitex negundo* L. (Verbenaceae) commonly known as Nirgundi. It is a large woody aromatic and multipurpose medicinal shrub. It is used medicinally throughout the greater part of India. This species is widely used in Chinese herbal medicine and is the second most important for treatment of chronic bronchitis. Leaf extract of this plant possess antibacterial and antitumor activity. In the present study, nodal segments of *Vitex negundo* were taken as source of explants and grown on MS media with 3% Sucrose and 0.8% agar-agar, supplemented with different concentrations of BAP, KIN (0.5 – 3.5 mg^l⁻¹) and TDZ (0.5-2.0), with various auxins (NAA, IBA, TIBA), incubated under a photoperiod of 16h illumination of light and 8h dark at 25±2°C. MS + 1 mg^l⁻¹ BAP was found to be the best concentration for shoot regeneration (90%). The regenerated shoots were sub-cultured for rooting, using different concentrations of IBA and NAA. Present optimized micropropagation protocol offers the possibility of germplasm conservation and mass cultivation of this important medicinal plant.

KEY WORDS: *VITEX NEGUNDO*, *REGENERATION*, *NODAL EXPLANT*, *CALLOGENESIS*

INTRODUCTION

Medicinal plants have been the subject of curiosity since times immemorial (Constable, 1990). Almost every civilization has a history of medicinal plant uses. About 80% of the people living in developing countries depend

on indigenous medicines to meet their primary health care needs. About 85% of these traditional medicines involve the consumption of plant extracts. Out of 250 species of the genus *Vitex*, near about 14 species have been found to occur in India. *Vitex negundo* L. (Verbenaceae) is a perennial aromatic, large woody shrub, tri or

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penta-foliolate leaves with purple color flower in branched tomentose cymes. It is commonly called as Chaste tree, Nirgundi (Hindi) and Monk's pepper. It is an important agro-forestry tree (200–300 cm high) found throughout the greater part of India and has been included in the list of valuable plant species due to its wide use in the Indian system of medicine (Kapur *et al.*, 1994). It possesses various medicinal properties (Muthuswamy *et al.*, 2012; Basri *et al.*, 2014; Bano *et al.*, 2015; Lad *et al.*, 2016).

The plant possesses anti-arthritic, hepatoprotective, anti-inflammatory, anti-allergic, insecticidal, antioxidant, antibacterial, immunomodulatory, antifungal as well as mosquito repellent activities (Islam *et al.*, 2013; Zheng *et al.*, 2014; Singh *et al.*, 2015; Lad *et al.*, 2015; Lad *et al.*, 2016). Leaves are aromatic, used as an antifertility drug (Bhargava, 1986) and possess snake neutralizing activities, (Minu *et al.*, 2012) (Muthuswamy *et al.*, 2012; Durairaj *et al.*, 2014) Dharmadasa *et al.*, (2016) also reported the anti-snake venom properties. Leaves are antiparasitic and used as alternative vermifuge and anodyne. They are also very effective to reduce inflammatory swellings of joints in rheumatism and relieve catarrh and headache. Root is used as tonic, diuretic and expectorant. It regulates hormones, enhances breast milk production and possesses progesterogenic properties as well (Au *et al.*, 2008; Arora *et al.*, 2011; Basri *et al.*, 2014; Haider *et al.*; 2017).

Betulinic acid, ursolic acid and β -sitosterol are some of its active constituents, isolated from its leaves which have been found to possess anti-cancer, anti-HIV and angiogenic properties, respectively (Basri *et al.*, 2014). In nature the species propagates through stem cutting and seeds. Based on our preliminary investigations propagation with vegetative cuttings is very slow and the survival rate is very limited. Propagation through seeds is hindered due to poor germination. Thus conventional propagation through seeds and vegetative cutting is not an adequate solution to meet the demand for this rare medicinal plant. Hence this study was carried out to develop an efficient protocol for its mass cultivation.

MATERIAL AND METHODS

Nodal explants were excised from elite plants of *Vitex negundo* growing in medicinal plants garden, School of Studies in Botany, Jiwaji University, Gwalior (M.P). The excised nodal explants of *V. negundo* were washed for 10 min under continuous stream of running tap water. Surface sterilization was done by treating the explants with 4% (v/v) Tween-20 (detergent; SRL, Pvt. Ltd, Mumbai, India) and rinsed with distilled water. These explants were then treated with 2% (w/v) bavistin solution (Systemic fungicide; BASP India Ltd., Mumbai India) for 5 min and followed by treatment with freshly prepared

0.1% HgCl_2 (SRL, Mumbai, India) for 3 min with continuous shaking under a laminar flow cabinet. These explants were finally washed 2-3 times by sterile distilled water prior to implantation in semisolid media.

The MS (Murashige and Skoog, 1962) basal medium was supplemented with 6-Benzylaminopurine (BAP), 6-Furfuryl-aminopurine (KIN), Thidiazuron (TDZ), Indole-3-butyric acid (IBA), 2,3,5-triiodobenzoic acid (TIBA), α -naphthalene acetic acid (NAA), at various concentrations and in various combinations for rhizogenesis. Full and half strength MS basal medium with IBA and NAA at different concentration was employed. All the plant growth regulators were procured from SRL and Himedia-Qualigens, SRL, Glaxo, CDH, Titan biotech and Himedia. 3% (w/v) sucrose (SRL, Mumbai, India) was used as Carbon source, solidified with 0.8% agar-agar and pH was adjusted to 5.75 using 0.1 N NaOH or 0.1 N HCl. 20 ml media (aprox.) was dispensed in each 150×25 cm test tube (Borosil, India), tightly covered with air tight plastic test tube caps and sterilized by autoclaving at 1.06 kg cm^{-2} at 121°C for 15 min. The explants were cultured in vertical orientation in test tubes containing semisolid medium. Cultures were maintained at 25±2 °C temperature with a relative humidity of 55±5 % under regular cycle of light (450-460 $\mu\text{W cm}^{-2}$) by cool day light emitted from fluorescent incandescent tubes (40 W, Philips & Finolex, India) of 16 hr light followed by 8 hr dark period.

After root formation, healthy plantlets with well developed root system were removed from medium and washed under running tap water to remove the medium. These are then transferred to plastic pots (5 cm diameter) containing autoclaved mixture of soil, sand and vermicompost (1:1:1). Subsequently acclimatization was achieved by covering the plastic pots with polythene bags to maintain humidity. Plants were irrigated with 1/10th of major salts of MS media. After 1 week, 3-5 holes are made in the poly bags. Plants were irrigated after every 5 days. The potted plants were maintained in the culture room. After 30 days the plantlets were potted in earthen pots with garden soil.

The shoot response of explants was evaluated after 35 days of culture in terms of percentage of explants producing shoots, average number of shoots per explant and average shoot length per explant. For root response, percentage of shoot producing roots, average number of roots per explant and average root length was recorded. All the values have been reported as mean value along with standard error (Mean ± SE).

RESULTS AND DISCUSSION

An ever increasing demand of uniform medicinal plants based medicines warrants their mass propagation through plant tissue culture strategy. Tissue culture technology is

Table 1. Effect of growth regulators on shooting during in-vitro culture of *Vitex negundo* L. on MS media.

Cytokinins (mg/l)		% of shoot induction	Average number of shoots per explant (Mean \pm SE)	Shoot Length (cm) (Mean \pm SE)
Control	0	10	0.12 \pm 0.01	0.72 \pm 0.01
BAP	0.5	80	3.48 \pm 0.34	2.64 \pm 0.59
	1.0	90	4.29 \pm 0.07	3.28 \pm 0.31
	1.5	80	2.59 \pm 0.37	2.01 \pm 0.27
	2.0	70	3.11 \pm 0.82	1.81 \pm 0.14
	2.5	50	2.40 \pm 0.30	1.78 \pm 0.07
	3.0	40	2.10 \pm 0.15	1.62 \pm 0.14
	3.5	30	2.00 \pm 0.03	1.50 \pm 0.18
KIN	0.5	70	2.43 \pm 0.03	1.86 \pm 0.18
	1.0	60	2.87 \pm 0.24	3.02 \pm 0.24
	1.5	80	3.47 \pm 0.14	3.33 \pm 0.08
	2.0	70	2.8 \pm 0.2	2.17 \pm 0.20
	2.5	60	2.62 \pm 0.18	1.81 \pm 0.34
	3.0	50	1.92 \pm 0.20	1.61 \pm 0.16
	3.5	40	1.64 \pm 0.07	1.63 \pm 0.16
TDZ	0.5	60	2.16 \pm 0.16	1.63 \pm 0.19
	1.0	50	2.4 \pm 0.28	1.46 \pm 0.06
	1.5	30	1.75 \pm 0.25	1.7 \pm 0.09
	2.0	20	2.33 \pm 0.33	1.43 \pm 0.12

potent and has opened extensive areas of research for biodiversity conservation. Tissue culture protocols have been developed for a wide range of medicinal plants, which includes endangered, rare and threatened plant species. (Sharma *et al.*, 2010). Conventional propagation methods are unable to meet the demand of the pharmaceutical industries and drug research. Therefore, it is necessary to develop a non-conventional method for propagation to fulfill the demands of the drug market (Rathore *et al.* 2008). *In vitro* propagation methods offer a powerful tool for conservation of germplasm and mass-multiplication of threatened plant species (Murch *et al.* 2000). It helps in micropropagation of large number of plant in shorter time period, irrespective of season and serves as an alternative source of plant propagation (Yadav and Singh, 2012; Yadav *et al.*, 2013; Groach *et al.*, 2014). This method can be employed in multiplying important endangered plant species which are difficult to propagate by conventional means and saves the plant from the extinction.

In order to establish an efficient *in vitro* micropropagation protocol for commercial exploitation of this plant, nodal explants of *V. negundo* were inoculated on MS medium supplemented with varied concentration (0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5 mg/l⁻¹) of cytokinins

(BAP, KIN) and TDZ (0.5-2.0 mg/l⁻¹). The nodal segments cultured on growth regulator free MS medium showed minimum signs of bud break even after 15 days of inoculation. The average number of shoots induced on MS basal medium was 10% with an average shoot length of 0.72 \pm 0.01 cm after 35 days of culture (Table 1).

However, addition of cytokinin was essential for differentiation of multiple shoots from the nodal explants. Of the three cytokinins tried, BAP was most effective over the other two for induction of multiple shoots. Similar effect have already been reported in various taxa like *Cassia angustifolia* (Agrawal *et al.*, 2002), *Spilanthes acmella* (Pandey and Agrawal 2009), *Aegle marmelos* (Yadav and Singh 2011), *Tylophora indica* (Faisal *et al.*, 2007), *Achyranthes aspera* (Ishwarya *et al.*, 2018), *Vitex trifolia* (Ahmad and Anis, 2014). The nodal segments responded by initial enlargement of dormant axillary buds followed by bud break within a week and multiple shoot induction and proliferation within 15 days of culture on BAP containing media. 1 mg/l⁻¹ BAP was optimum in inducing 90% morphogenic culture with an average of 4.29 \pm 0.07 shoots per explant having an average shoot length of 3.28 \pm 0.31 cm after 35 days of culture (Table 1, Fig.1A). BAP at 3.5 mg/l⁻¹ displayed poor morphogenic response both in terms of average number of shoots and average

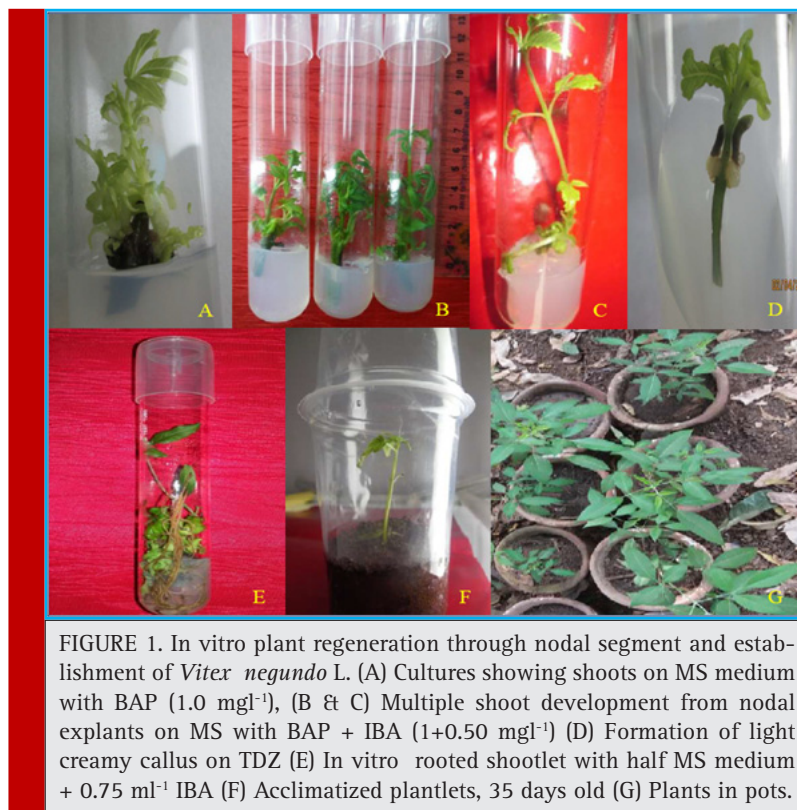


FIGURE 1. In vitro plant regeneration through nodal segment and establishment of *Vitex negundo* L. (A) Cultures showing shoots on MS medium with BAP (1.0 mg l⁻¹), (B & C) Multiple shoot development from nodal explants on MS with BAP + IBA (1+0.50 mg l⁻¹) (D) Formation of light creamy callus on TDZ (E) In vitro rooted shootlet with half MS medium + 0.75 ml⁻¹ IBA (F) Acclimatized plantlets, 35 days old (G) Plants in pots.

Table 2. Effect of BAP 1 mg l⁻¹ and 1.5 mg l⁻¹ KIN in combination with different concentrations of auxins on shoot bud regeneration from nodal explants of *Vitex negundo*

Concentration of growth regulators in mg l ⁻¹	IBA	TIBA	Percentage of explants producing shoots (%)	Number of shoots per explant (Mean ± SE)	Shoot Length (cm) (Mean ± SE)
BAP 1	0.25		60	2.66 ± 0.33	3.23 ± 0.09
	0.50		90	6.12 ± 0.63	3.85 ± 0.21
	0.75		70	3.14 ± 0.40	3.78 ± 0.39
		0.25	50	2.8 ± 0.37	3.02 ± 0.17
		0.50	70	3.5 ± 0.42	3.53 ± 0.08
		0.75	60	2.5 ± 0.34	3.28 ± 0.10
KIN 1.5	0.25		70	4.37 ± 0.41	2.83 ± 0.26
	0.50		80	5.88 ± 0.38	4.13 ± 0.43
	0.75		60	4.16 ± 0.60	3.18 ± 0.17
		0.25	60	4.33 ± 0.42	3.21 ± 0.20
		0.50	80	5.2 ± 0.53	3.71 ± 0.15
		0.75	70	4.38 ± 0.47	3.32 ± 0.12

Results were recorded after 35 days and are presented as Mean ± Std. Error.

Table 3. Effect of growth regulators on rooting pattern of *Vitex negundo* L. during *in-vitro* culture (full and ½ strength of MS media)

Growth regulator (mg/l)		Percentage of explants producing roots (%)	Average number of roots per explants	Average root length (cm)
MS Full + IBA	0.50	70	5.71 ± 0.71	1.88 ± 1.20
	0.75	80	6.75 ± 0.52	2.33 ± 2.21
	1.0	60	4.33 ± 0.42	1.86 ± 2.10
MS half + IBA	0.50	80	7.57 ± 0.92	3.25 ± 3.76
	0.75	90	12.12 ± 0.83	4.98 ± 2.50
	1.0	70	8.9 ± 0.89	4.53 ± 3.92
MS full + NAA	0.50	70	4.85 ± 0.76	1.32 ± 0.77
	0.75	80	6.25 ± 0.59	2.23 ± 2.62
	1.0	60	4.33 ± 0.42	1.71 ± 3.37
MS half + NAA	0.50	70	6.8 ± 0.86	1.74 ± 0.07
	0.75	90	8.42 ± 0.89	2.66 ± 0.30
	1.0	60	7.33 ± 0.66	1.95 ± 0.13

shoot length (Table 1). On increasing the concentration of BAP, induction of multiple shoots was comparatively low and average shoot length too decreased (Table 1). Except BAP, all the tried concentrations of KIN and TDZ showed poor morphogenic response in term of average number of induced shoots and shoot length (Table 1). Considerable callusing at the basal cut end of nodal segment along with formation of multiple shoots was also reported in the present study which agrees with the study on *Azadirachta indica* (Arora *et al.*, 2010) which showed similar results (Fig.1 D). The formation of callusing at the basal cut ends of nodal segment due to the action of accumulated auxins at the basal cut proliferation, especially in the presence of cytokinins (Marks and Simpson, 1994). The present study also revealed the synergistic effect of BAP in combination of auxin for effect shoot regeneration which has also been reported in studies of *Celastrus paniculatus* (Lal *et al.*, 2010).

The highest number of shoots (6.12±0.63) developed was observed in MS with BAP 1 + IBA 0.50 mg/l⁻¹ (Table 2 Fig.1 B & C). The highest proliferation rate (90%) was also found at the same combination of plant growth regulators in the medium.

The best results were observed on a medium containing BAP and IBA which is supported by earlier studies in *Chonemorpha grandiflora* (Nishitha *et al.*, 2006), *Vitex negundo* (Ahmad and Anis, 2011), *Launaea cornuta* (Ambajo and Matheka, 2016), *Mimosa pudica* (Bianchetti *et al.*, 2017) *Tylophora indica* (Najar *et al.*, 2018), *Ceropegia juncea* (Binish, 2018), In these stud-

ies also synergistic effects were observed when Cytokinin was used in combination with auxin. Among the two different types of auxins employed for root induction on *in vitro* excised shoots of *V. negundo*, IBA was found to be most effective. A maximum of 90% shoots induced an average of 12.12±0.83 roots with an average root length of 4.98±2.50cm after 3 weeks on half strength MS medium augmented with 0.75 mg/l⁻¹ IBA (Table 3). The roots were induced directly from the shoot base without callus formation at this concentration. (Table 3, Fig.1 E). Similar responses have been already reported in *Spilanthes acmella* (Pandey and Agrawal 2009, Yadav and Singh 2010), (Reddy *et al.*, 2014), *Ceropegia juncea* (Binish, 2018). However, at higher concentration of IBA, the number of roots and root length showed decline. Compared to IBA, poor rooting response was observed at the concentration of IBA + full MS and NAA + full and half MS. The tissue culture derived plantlets (Fig.1 F & G) were acclimatized in the field condition with 90% survival. Such micropropagated plants were found to be morphologically similar to the mother plant.

An efficient protocol has been developed for regeneration of *Vitex negundo* which offers a great potential to cater the needs of different pharmaceutical industries. In the present study, enhanced *in vitro* regeneration of plants with combination of plant growth regulators such as BAP, KIN, TDZ, IBA and TIBA was observed. This will be helpful in understanding the callogenesis and organogenesis through the nodal explants and to facilitate the mass propagation of *Vitex negundo*.

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