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In vitro Regeneration of *Vitex negundo* L. - A Multi-purpose Woody Aromatic Medicinal Shrub

M. Jawahar*, S. Ravipaul[1] and M. Jeyaseelan[2]

PG Department of Botany, Government Arts College, Karur, Tamil Nadu, India

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Abstract

A rapid and efficient protocol was developed for inducing indirect organogenesis using leaf explants of *Vitex negundo* L. Explants were cultured on MS with different concentrations of 2,4-D and IAA in combination with BAP for callus induction. The frequency of callus induction increased with increasing concentration of IAA (0.3 mg/l) and BAP (0.3 mg/l) at optimal level. The shoot buds appeared emerging as green coloured protuberances on the callus. The high frequency of shoot bud initiation and shoot proliferation was observed on MS containing 0.3 mg/l IAA and 0.3 mg/l BAP. The regenerated shoots were successfully rooted on MS supplemented with 0.5 mg/l IBA. Rooted plants were transferred to pots containing sand, soil and manure in the ratio of 1 : 1 : 1. Nearly 90% survival of *in vitro* plants were recorded.

Introduction

Vitex negundo L. is a large woody aromatic and multipurpose medicinal shrub belonging to the *Verbenaceae* (Wealth of India 1976). This species is widely used in Chinese herbal medicine and it is the second most important treatment for chronic bronchitis. They are useful in dispersing swellings of the joints from acute rheumatism, and of the testes from suppressed gonorrhea. The juice of the leaves is used for removing fetid discharges and worms from ulcers, whilst oil prepared with the leaf juice is applied to sinuses and scrofulous sores. The stem decoction is used in the treatment of burns and scalds. The fresh berries are pounded to a pulp and used in the form of a tincture for the relief of paralysis, pains in the limbs, weakness etc. The plant is said to be material preventative and is also used in the treatment of bacterial dysentery. Extracts of the leaves have shown bacterial dysentery. Extracts of the leaves have shown bactericidal and

*Corresponding author, E-mail: somajainn@yahoo.co.in, [1]PG & Research Department of Biotechnology, Bharath College of Science and Management, Thanjavur,Tamil Nadu, India. [2]Department of Biotechnology, Ponnaiyah Ramajayam College, Thanjavur, Tamil Nadu, India.

anti tumor activity (Chopra et al. 1986). The principal constituents of *V. negundo* is casticin, isoorientin, chrysophenol D, luteolin, P-hydroxybenzoic acid and D-fructose. Herbal medicines are the precursors of many common drugs prescribed in clinical practice in countries today. Furthermore, herbs and herbal products are still an important part of the primary health care systems in many parts of the world.

Medicinal plants are of great interest to the researches in the field of biotechnology as most of the drug industries depend, in part, on plants for the production of pharmaceutical compounds (Chand et al. 1997). *In vitro* culture techniques which offer a viable tool for mass multiplication and germplasm conservation of rare, endangered, aromatic and medicinal plants (Arora and Bhojwani 1989, Sharma et al. 1991, Sudha and Seeni 1994, Sahoo and Chand 1998, Karuppusamy and Pullaiah, 2007, Jawahar et al. 2008). This paper describes an efficient protocol for callus induction and plant regeneration from leaf explants of *V. negundo*.

Materials and Methods

Healthy young leaf explants were collected from three - five months old plants of *V. negundo* L. growing in the college herbal garden. After trimming of the larger leaves, explants were washed under running tap water followed by treatment with a surfactant, Tween 20 (5% v/v) for 10 min. The explants were further treated with 70% ethanol for 10-15 sec, followed by 5-10 min in double distilled water, surface sterilization was done with mercuric chloride (0.1% w/v) solution for 2-3 min. Then the explants were finally rinsed four - five times with sterile distilled water and inoculated on MS supplemented with various concentrations of growth regulators. Sucrose (30 g) and 0.8% agar were used. After adjusting the pH (5.8), the medium was autoclaved at 121^{o}C for 15 minutes. The cultures were given illumination by white fluorescent light with an intensity of 50 μmol $m^{-2}s^{-1}$ and maintained at 25 ± 2^{o}C under 16 : 8 light and dark regime. All the treatments were repeated at least three times with 30 replicates and data were subjected to statistical analysis.

Results and Discussion

The leaf explants obtained from young plants were used in this study to optimize salts, growth regulators, organic supplements, complex extracts and amino acids and their concentration. MS fortified with 2, 4-D and IAA in combination with BAP was investigated for callus induction. The morphogenic responses of explants were also studied.

The leaf explants were cultured on MS supplemented with different concentrations of auxins 2, 4-D and IAA (0.1 to 0.5 mg/l) in combination with 0.3 mg/l BAP for callus induction. The explants remained green and fresh. Moreover, explants enlarged two - three times compared with original size and green compact callus initiation occurred one week after inoculation. The higher frequency of callus proliferation was observed on leaf explants in three - four weeks old cultures (Fig. 1a). The morphogenic responses of explants to various

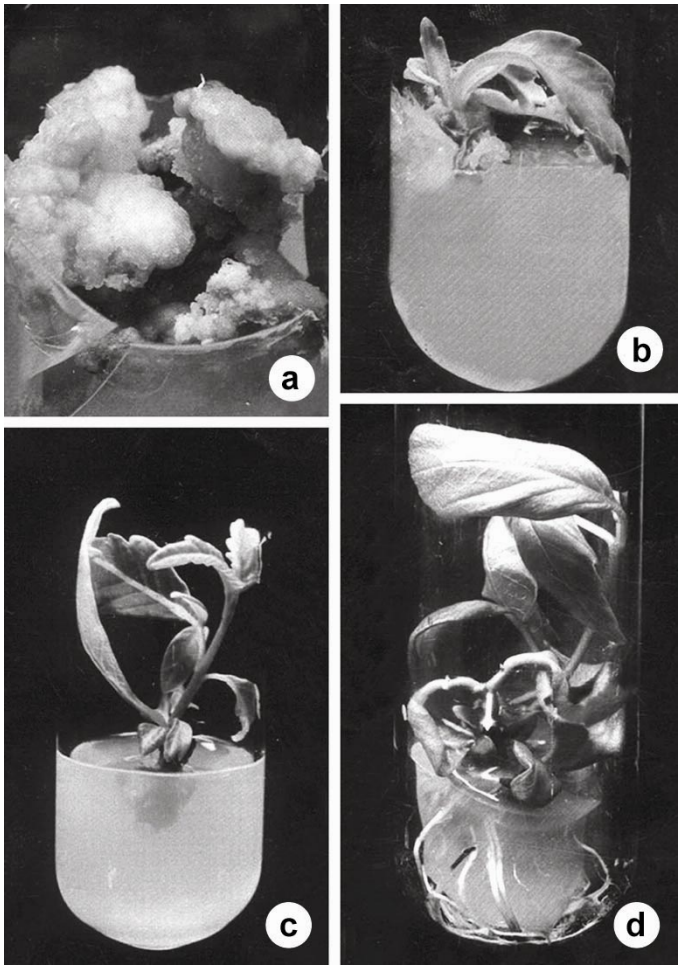

Fig. 1. *In vitro* callus and shoot multiplication of *Vitex negundo*. (a) Callus proliferation. (b) Multiple shoot proliferation. (c) Shoot elongation and (d) Root induction.

growth regulators such as 2, 4-D and IAA in combination with BAP are presented in Table 1. Among the different concentrations and combinations used 0.3 mg/l IAA with 0.3 mg/l BAP induced a higher frequency of callus proliferation i.e. 80%. There was a significant variation in percentage of response above and below the optimal concentration of growth regulators. Callus growth

was best in the medium containing 0.3 mg/l IAA + 0.3 mg/l BAP followed by 0.3 mg/l 2, 4-D + 0.3 mg/l BAP. Similar reports were reported in *Cardiospermum halicacabum* (Jawahar et al. 2008), and in *Vitex negundo* (Thiruvengadam and Jayabalan 2000).

Table 1. Effect of 2,4-D, IAA and combination with 0.3 mg/l BAP for *in vitro* callus induction from leaf explants of *Vitex negundo*.

Growth regulators (mg/l)	% of callusing	% of shoot proliferation	No. of multiple shoots	Shoot length (cm)
2,4-D				
0.1	30.3	25.0	3.07 ± 0.41^{j}	3.47 ± 0.25^{h}
0.2	55.0	45.0	4.88 ± 0.30^{h}	5.76 ± 0.15^{de}
0.3	70.0	60.0	6.96 ± 0.48^{e}	7.80 ± 0.10^{b}
0.4	60.0	52.0	5.54 ± 0.43^{g}	5.50 ± 0.26^{de}
0.5	50.0	40.0	3.98 ± 0.14^{i}	4.70 ± 0.36^{g}
IAA				
0.1	40.0	30.0	6.24 ± 0.15^{ef}	5.13 ± 0.20^{f}
0.2	60.0	55.0	11.62 ± 0.93^{c}	6.33 ± 0.56^{c}
0.3	80.0	70.0	$17.39 \pm .71^{a}$	9.40 ± 0.52^{a}
0.4	65.0	55.0	12.99 ± 1.36^{b}	7.03 ± 0.75^{bc}
0.5	45.0	45.0	9.63 ± 0.88^{d}	5.86 ± 0.30^{d}

Each value represents the mean ± Sd of 30 replicates and each experiment was repeated at least thrice. Values with the same superscript are not significantly different at the 0.05% probability level according to DMRT.

The regeneration of adventitious shoots from leaf callus was dependent on both auxin and cytokinin. Multiple shoot buds were visible approximately after five weeks of culture on same medium. The maximum number of shoot bud initiation was observed in five to seven week old cultures. Among the different concentrations and combinations used, 0.3 mg/l IAA + 0.3 mg/l BAP induced higher frequency of multiple shoot proliferation and shoot numbers i.e. 70% and 17.39 ± 0.71 followed by 0.3 mg/l 2,4-D + 0.3 mg/l BAP (Table 1, Fig 1b). Above the optimal level of IAA + BAP and 2,4-D + BAP a reduction in the multiple shoot proliferation was noticed. The presence of auxin and cytokinin in the culture medium promoted higher frequency of shoot proliferation and shoot elongation in *Vitex negundo* L. (Fig. 1c). The potential for multiple shoot proliferation and shoot elongation appears to strong in the presence of lower concentration of auxin and in combination with cytokinin in *Vitex negundo* L. NAA combined with BAP has been reported the best shoot proliferating combination in *Heracleum candicans* (Wakhlu and Sharma 1999), *Centella asiatica* (Shashikala et al. 2005) and *Cardiospermum halicacabum* (Jawahar et al. 2008). In

contrast, Fraternale et al. 2002) reported that high concentration of auxin with cytokinin was suitable for shoot multiplication in *Bupleurum fruticosum*.

Regenerated shoots (3 cm and above in length) were excised and placed on MS supplemented with various concentrations of IAA and IBA (0.1 to 0.5 mg/l) for root induction. Optimal rooting and growth of microroots were observed without intervening callus 7 - 10 days after transfer. The percentage of root formation and the number of roots per shoot significantly varied depending on concentrations of IAA and IBA (Table 2). The higher frequency of rooting (82.3%)

Table 2. Effect of IAA and IBA on root induction in regenerated plantlets.

IAA + IBA (mg/l)	% of root induction from shoots	Av. number of roots/shoot
IAA		
0.1	28.3	4.66 ± 0.57^{hi}
0.2	35.0	5.66 ± 0.57^{fg}
0.3	46.6	7.00 ± 1.00^{c}
0.4	58.3	8.66 ± 0.57^{cd}
0.5	75.0	10.38 ± 1.16^{b}
IBA		
0.1	30.0	5.00 ± 1.00^{h}
0.2	45.0	6.33 ± 0.57^{f}
0.3	53.3	7.00 ± 1.00^{e}
0.4	64.5	9.00 ± 0.57^{c}
0.5	82.3	11.60 ± 0.57^{a}

Each value represents the mean ± Sd of 30 replicates and each experiment was repeated at least thrice. Values with the same superscript are not significantly different at the 0.05% probability level according to DMRT.

with highest root numbers (11.60 ± 0.57) was obtained in medium containing 0.5 mg/l IBA followed by 0.5 mg/l IAA (Fig. 1d). However, there were no significant differences between the effect of IBA and IAA. Similar results were reported by Jawahar et al. (2008) in *Cardiospermum halicacabum*. Sunichan et al. (1998) reported that IBA was effective for root induction in *Sterculia urens.* Quarishi and Mishra (1998) obtained rooting response in IAA. Pattnaik and Chand (1996) obtained best rooting on the medium containing IBA. In most of the plant species IBA and IAA are considered as the most effective growth regulators for the induction of roots. Wakhlu and Sharma (1999) reported that the medium containing IBA produced maximum number of adventitious roots in *Heracleum candicans*. The rooted plants were first transferred to plastic cups having vermiculite and garden soil (3 : 1). The plastic cups were covered with polythene pack and kept for a week in a culture room at 25 ± 2°C under 16 h photoperiod. After a week, these were transferred to the green house and then to the field.

From our experimental data, it is evident that IAA combined with BAP induced higher frequency of callus, shoot proliferation and shoot elongation and IBA induced a higher frequency of rooting in leaf explants of *V. negundo* L. In conclusion, this organogenesis and shoot multiplication system is suitable for conservation of germplasm of this multipurpose medicinal plant.

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