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In vitro studies on *Vitex negundo*, a potent medicinal plant

Rameshwar Groach, Kuldeep Yadav, Narender Singh*

Plant Tissue Culture Laboratory, Department of Botany, Kurukshetra University, Haryana 136119, India

*Corresponding author, E-mail: nsheorankukbot11@gmail.com

Abstract

An efficient plant regeneration protocol for *in vitro* propagation of *Vitex negundo* L., an important medicinal plant, was developed using mature nodal explants. Multiple shoot formation was significantly influenced by growth regulators, carbon source and type of solidifying agent used. Among the various combinations used, Murashige and Skoog medium with 2.0 mg L^{-1} 6-benzylaminopurine + 0.5 mg L^{-1} α-naphthalene acetic acid, containing 25% sugarcane juice and 0.8% agar, exhibited highest bud-forming capacity (93.3%) with maximum number of formed shoots per explant (4.14). Maximum root induction response (66.6 %) was observed on Murashige and Skoog half strength semisolid medium supplemented with 1.0 mg L^{-1} indole-3-butyric acid. The regenerated plantlets were successfully transferred to pots containing sterilized soil and sand (3:1) mixture and acclimatized with >70% survival rate under field conditions.

Key words: carbon source, solidifying agents, micropropagation, multiple shoots, *Vitex negundo*.
Abbreviations: BAP, 6-benzylaminopurine; IBA, indole-3-butyric acid; NAA, α-naphthalene acetic acid.

Introduction

Vitex negundo L. (Verbenaceae), commonly known as Nirgundi, Tarvan, Sephali and Sambhalu, is an important woody, agro-forestry tree (200 to 300 cm high) in east Asia, south west China and tropical Africa (Kapur et al. 1994). All the parts of the plant are medicinally significant and possess astringent, stomachic, cephalic and anthelmintic properties (Choi et al. 2002) and snake neutralizing activity (Alam, Gomes 2003). Some of the active constituents isolated from its leaves like betulinic acid, ursolic acid and β-sitosterol, possess antifeedent, antibacterial, anti-cancer, anti-HIV and angiogenic properties (Chandramu et al. 2003). It also produces root suckers, and thus is used for planting against soil erosion and for afforestation, especially in stabilization of forest lands affected by floods (Anonymous 2003).

Unrestricted removal and overexploitation of this medicinal plant for the preparation of various valuable medicines has caused drastic reduction of this important genetic resource in India. The conventional method for propagation of *V. negundo* is through seeds or root suckers. However, poor seed germination potential and the dependence of a root sucker on plant age restrict its multiplication (Sahoo, Chand 1998). Micropropagation is a better alternative to conventional vegetative propagation, mainly for raising of elite species with conservation of space and time (Yadav et al. 2013a).

Although, there are many reports on the *in vitro* propagation of *V. negundo* (Rani, Nair 2006; Ahmad, Anis 2007; Chandramu et al. 2003), considerable efforts are still required to make it more economical and practical. In the light of the above-referred importance and demand, the present investigation was undertaken to evaluate the effect of different growth regulators, carbon sources and solidifying agents to establish an efficient protocol for *in vitro* multiplication of *V. negundo*.

Materials and methods

Explant collection and disinfection

Nodal explants were collected from mature plants growing in Herbal Garden of Botany Department, Kurukshetra University, Haryana (India). These were washed under running water with Tween-20 (two drops per 100 mL water) and sterilized with 0.1% (w/v) mercuric chloride for 3 to 5 min and then given a dip in 70% ethanol. Nodal explants were rinsed with sterile distilled water four to five times to remove traces of mercuric chloride.

Culture media and conditions

Murashige and Skoog (1962; MS) medium supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar was used in the present study. The pH of the medium was adjusted to 5.8 by using 0.1 N NaOH and 0.1 N HCl before autoclaving at 120 °C for 20 min. After the inoculation, the culture tubes were incubated at 25 ± 2 °C under 16 h photoperiod with a photosynthetic photon flux density of 40 μmol m^{-2} s^{-1} and 60 to 70% humidity. All aseptic manipulations were carried out under a laminar airflow chamber.

Effect of various carbon sources, i.e. sucrose (3%), table sugar (3%) and sugarcane juice (25%), along with different solidifying agents, i.e. agar (0.8 %) and sago powder (13% w/v) on MS medium containing 2.0 mg L^{-1} 6-benzylaminopurine (BAP) + 0.5 mg L^{-1} α-naphthalene

acetic acid (NAA) on *in vitro* growth of *V. negundo* was also tested. Sago is a powdery starch derived from tuber of *Manihot esculenta*.

Shoot induction and multiplication

The surface sterilized explants were trimmed gently with a sterilized blade to remove the sterilizing agent-affected brown parts and inoculated on MS media supplemented with various concentrations (0.5 to 2.0 mg L^{-1}) of BAP alone and in combination with 0.5 mg L^{-1} NAA for culture initiation. The explants producing shoots were subcultured onto fresh media after every four weeks. A set of explants on MS medium without growth regulators served as controls.

In vitro *rooting and acclimatization*

The *in vitro* regenerated shoots (2.5 to 3.0 cm) were excised aseptically and implanted on half strength MS medium without or with various concentrations (0.5 to 2.0 mg L^{-1}) of indole-3-butyric acid (IBA) for rhizogenesis.

The rooted plantlets were taken out from rooting medium and washed several times with sterile distilled water to remove the traces of agar. These plantlets were then transferred to pots containing sterile soil:sand (3:1). Potted plantlets were covered with transparent plastic bags to ensure high humidity and watered every two days with half strength MS salt solution for two weeks. Thereafter, bags were removed in order to acclimatize the plantlets to field conditions. After four weeks, acclimatized plantlets were transferred to pots containing normal garden soil and maintained in a greenhouse under normal day length conditions.

Statistical analysis

All the experiment were conducted with a minimum of five replicates per treatment and were repeated three times. The data were analyzed statistically using one-way analysis of variance (ANOVA) and the differences contrasted using a Duncan's multiple range test at $P \leq 0.05$. All statistical analyses were performed using the SPSS (version 11.5) program.

Results and discussion

MS basal medium without growth regulators did not show any response. The results showed that BAP alone at higher concentration or in combination with NAA was more effective for shoot formation as compared to other lower concentrations (Table 1). The highest degree of shoot initiation was observed on MS medium supplemented with 2.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA (Fig. 1b). The same medium also showed maximum (86.6 %) bud break after 2.0 mg L^{-1} BAP (Table 1; Fig. 1A and B). Further, decrease of this concentration not only decreased the percent bud break, but also decreased the number of shoots produced. Similar reports about the effectiveness of BAP on shoot multiplication has been reported by many workers, e.g. Kaur et al. (1992) in *Anogeissus sericea*, Perezeparron et al. (1994) in *Fraxinus angustifolia* and Mao et al. (2000) in *Litsea cubeba*. Lal et al. (2010), Verma et al. (2011) and Yadav and Singh (2012) also noted the synergistic effect of BAP in combination with an auxin for efficient shoot regeneration. NAA regulates not only vegetative growth but also organ growth, whereas BAP facilitates cell division and sprouting (Pan 2001).

Carbohydrates act as an energy source required for growth, maintenance and synthesis of cell constituents during *in vitro* culture. The most commonly used carbon source is sucrose, but other sugars like glucose, fructose, dextrose, mannitol, sorbitol etc. are also occasionally used. Sul and Korban (1998) reported that the carbon source and their concentrations in a culture medium also affect the *in vitro* growth of plants. Sucrose is required for differentiation of xylem and phloem elements in cultured cells (Aloni 1980). It also represents the major osmotic component of the medium and necessary for various metabolic activities. In most plants, 2 to 3% sucrose is found to be very effective for optimal growth and morphogenesis. In our study, sugarcane juice was found to be a good alternative to laboratory sucrose. Sugarcane juice at 25% concentration not only resulted in maximum bud break, but also generated higher number of shoots per nodal explant (Table 2; Fig. 1C). Plants cultured on sucrose grew taller than those cultured on sugarcane juice. This may be due to the fact that sugarcane juice also contains other reducing sugars apart from sucrose, which are known to speed up cell division leading to an increase in the volume and weight of tissues. It also contains other elements like iron, phosphorus, potassium and sodium in comparison to

Table 1. Effect of various concentrations of BAP alone and in combination with NAA on shoot initiation from mature nodal explants of *V. negundo* after 30 days of culture. Mean values followed by different letters within a column do not differ significantly at $P \leq 0.05$ according to Duncan's Multiple Range Test

Plant growth regulators (mg L^{-1})	Bud break (%)	Number of shoots	Shoot length (cm)
BAP (0.5)	40.0d	1.33c	1.60e
BAP (1.0)	66.6c	2.60b	2.40c
BAP (2.0)	80.0b	3.33ab	3.08a
BAP (1.0) + NAA (0.5)	80.0b	3.00b	2.32d
BAP (2.0) + NAA (0.5)	86.6a	4.00a	2.82b

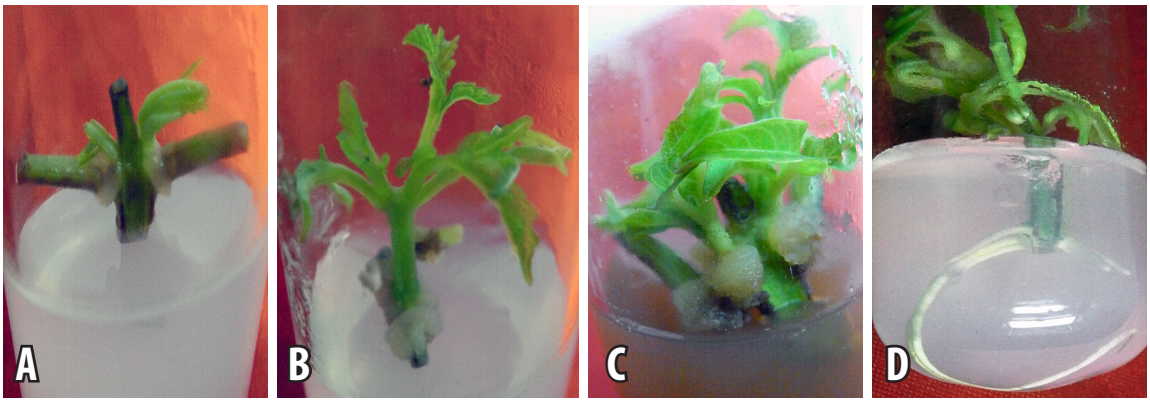

Fig. 1. A, initiation of multiple shoots derived from nodal explant on MS + BAP (2.0 mg L^{-1}) + sucrose (3.0%) + agar (0.8%). B, clump of proliferating shoots obtained on MS + BAP (2.0 mg L^{-1}) + NAA (0.5 mg L^{-1}) + sucrose (3.0%) + agar (0.8%). C, shoot proliferation on BAP (2.0 mg L^{-1}) + NAA (0.5 mg L^{-1}) + agar (0.8%) + sugarcane juice (25%). D, *in vitro* rooting on ½ MS + IBA (1.0 mg L^{-1}).

the scant traces in sucrose (Demo et al. 2008).

Agar is one of the most expensive and common gelling agents used to solidify medium in tissue culture media and assumed to provide neutral support for callus growth and multiplication (Prakash 1993). Gelling agents are usually added to culture medium to increase its viscosity as a result of which plant tissues and organs remain above the surface of the nutrient medium (Prakash et al. 2002). Agar is one of the major components that is replaced with low cost gelling alternatives to achieve cost reduction. Mostly 0.8% agar is used for culture medium. Exclusive use of agar results in over exploitation of this resource and makes it essential to look for other alternative and cheap sources to make tissue culture techniques economically feasible (Deb, Pongener 2010). Many workers have tested a few low-cost gelling agents viz., sago powder, isabgol husk, guar gum, cassava flour and xantham gum to replace agar (Babbar et al. 2005; Deb, Pongener 2010). Naik and Sarkar (2001) also used sago powder as a cheaper gelling agent for potato regeneration. Of the two gelling agents tested, agar was found to be the best media gelling agent (Table 2). In our case, the performance of this low cost gelling agent was found to be satisfactory and results compared well with agar. The difference in growth between agar and sago was possibly due to the differential osmotic effects, in addition to their varying nutritional supplement. Both, being of plant origin, are biodegradable and do not pose any threat to the environment.

Production of plantlets with profuse rooting under *in vitro* is an important step for successful establishment of regenerated plants in soil. However, in the present investigation excised shoots failed to develop roots on both full and half strength MS medium without growth regulators. MS medium lacking any plant growth regulators proved to be completely incompetent for root induction (Lal et al. 2010; Verma et al. 2011; Yadav, Singh 2011a; 2011b). In our study, half-strength MS medium with 1.0 mg L^{-1} IBA showed maximum root induction (Table: 3; Fig. 1D). IBA was also found to be effective for root induction in different plant species like *Commiphora mukul* (Singh et al. 2010), *Spilanthes acmella* (Yadav, Singh 2010) and *Stevia rebaudiana* (Verma et al. 2011).

The most crucial step in the micropropagation is hardening and acclimatization as this process provides plantlets capable of tolerating the external environmental conditions and survives. Various types of substrates have been used during acclimatization, such as soil vermiculite mixture, sterilized sand soil mixture and other biofertlizers like arbuscular mycorrhyzal fungi, *Pseudomonas* etc. (Philomina, Rao 1999; Thakur et al. 2001; Yadav et al. 2013b). The *in vitro* regenerated plantlets transplanted to small earthen pots containing sterilized soil and sand

Table 2. Effect of different carbon sources and solidifying agents on *in vitro* growth of *V. negundo* plantlets on MS medium containing 2.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA after 30 days. Mean values followed by different letters within a column do not differ significantly at $P \leq 0.05$ according to Duncan's Multiple Range Test

Carbon source	Solidifying agent	Bud Break (%)	Number of Shoots	Shoot length (cm)
Sucrose (3.0 %)	Agar (0.8%)	86.6b	4.00ab	2.82a
Table sugar (3.0 %)	Agar (0.8%)	80.0c	3.33bc	2.76ab
Sugar cane juice (25 %)	Agar (0.8%)	93.3a	4.14a	2.36c
Sucrose (3.0 %)	Sago powder (13%)	80.0c	3.00c	2.60b
Table sugar (3.0 %)	Sago powder (13%)	73.3d	2.80c	2.40c
Sugar cane juice (25 %)	Sago powder (13%)	80.0c	3.33bc	2.24c

Table 3. Root formation on different concentrations of IBA in *V. negundo* after 30 days. –, no response, Mean values followed by different letters within a column do not differ significantly at $P \leq 0.05$ according to Duncan's Multiple Range Test

Media composition (mg L^{-1})	Rooting (%)	Number of roots	Remarks
MS full strength without growth regulators	–	–	–
MS half strength without growth regulators	–	–	–
MS half strength + 0.5 IBA	40.0c	5.33c	Long and thin
MS half strength + 1.0 IBA	66.6a	7.40a	Long and thin
MS half strength + 2.0 IBA	53.3b	6.25b	Long and thin

mixture (3:1), covered with transparent polythene bags, showed maximum survival rate. After successful hardening, plantlets were transferred to pots containing normal soil and maintained in a greenhouse under normal day length conditions. Successful acclimatization and field transfer of *in vitro* regenerated plantlets have also been reported by Sivakumar and Krishanamurthy (2000), Lattoo et al. (2006) and Vadodaria et al. (2007).

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