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High Frequency Induction of Multiple Shoots from Nodal Explants of *Vitex negundo* using Silver Nitrate

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ABSTRACT

The effect of silver nitrate 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 (98.57%) of explants for shoot induction and multiple shoot (22.45 explant^{-1}) production were observed in the combination treatment of N-Benzyl adenine (BA) (16.80 μm L^{-1}), Indole-3-butyric acid (IBA) (2.25 μm L^{-1}) and 5% sucrose supplemented with 100 mg L^{-1} silver nitrate.

Key Words: *Vitex negundo* L.; Medicinal plant; Multiple shoots; Nodal explants; Silver nitrate

INTRODUCTION

Despite its economic importance, the production of *Vitex nugondo* L. is threatened by population growth, desertification, industrial development and attack by numerous parasites. The classical conservation techniques such as crossing, sexual and somatic hybridization and breeding give a genetic blind mixture. These techniques are limited by the sterility of the descents, the genetic barrier between species and the long life cycle of certain trees (Sederoff *et al.*, 1995). Plant tissue culture offers many unconventional techniques for plant improvement. *V. negundo*, a fast growing, multipurpose tree used for ornamental and roadside planting, belongs to a member of verbenaceae. Several parts of the plant are employed for antipyretic, analgesic, anti-asthma and antiseptic purposes by certain aboriginal people. The methanolic root extracts of *V. negundo* significantly antagonized the *Vipera russellii* and *Naja kaouthia* venom induced lethal activity both *in vitro* and *in vivo* studies (Alam & Gomes, 2003). The water extracts of *V. negundo* (aerial part) showed HIV-1 RT inhibition ratio (% IR) higher than 90% at a 200 μg. mL^{-1} concentration (Woradulayapinij *et al*., 2005). The plant was reported to be potent and novel therapeutic agents for scavenging of NO and the regulation of pathological conditions caused by excessive generation of NO and its oxidation product, peroxynitrite (Jagetia & Baliga, 2004). Bioassay-guided fractionation of the chloroform-soluble extract of the leaves of *V. negundo* led to the isolation of the known flavone vitexicarpin, which exhibited broad cytotoxicity in a human cancer cell line panel (Diaz *et al*., 2003). The $CHCl_3$ extract of the defatted seeds of the plant exhibited anti-inflammatory activity and yielded triterpenoids (Chawla *et al*., 1992). Moreover, *V. negundo* showed significant antibacterial activity against *Escherichia coli*, *Klebsiella aerogenes*, *Proteus vulgaris* and *Pseudomonas aerogenes* (gram-negative bacteria), which confirmed the traditional therapeutic claims for these herbs (Perumal Samy *et al*., 1998). But over looking all its medicinal importance, the plant is categorized as shrub. Hence, the effect of silver nitrate on systematic propagation of this medicinally important species was undertaken for the first time through micropropagation method. Micropropagation has many advantages over conventional propagation of plants (Stushnoff & Fear, 1985) and is important for the regeneration following transformation (Ainsley *et al*., 2000) and cryopreservation (Channuntapipat *et al*., 2000). Already, several species of plant have been manipulated in culture using nodal segments on MS full-strength medium fortified with various plant growth regulator combination. These included *Holostemma annulare* (Sudha *et al*., 1998), *Hemidesmus indicus* (Sharma & Yelne, 1995) and *Holostemma ada-kodien* (Martin, 2002). Moreover, the use of silver nitrate in established media induced enhanced organogenesis *in vitro* was achieved on *Rotula aquatica* L. (Chithra *et al*., 2004). But the effect of silver nitrate on shoot induction and multiple shoot formation from nodal explants of *V. negundo* is not

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

MATERIALS AND METHODS

Actively growing and healthy shoot material of *V. negundo*, with three to four nodes, were collected from an adult plant growing in the Putia, Rajshahi, Bangladesh. About 10.4 to 6 cm long shoot tips, with leaves attached were placed into small (10 x 20 cm) plastic bags. These were sealed and kept on ice. The shoot tips were processed within 4 h of collection. After removing the leaves, the shoots were cut into pieces (0.5-1.0 cm), each containing a single node with dormant axillary buds. The nodal segments were washed in 5% Teepol solution for 10-15 min followed by disinfecting in 0.1% mercuric chloride ($HgCl_2$) solution for 5 min in sterilized autoclaved bottles and finally washed three times with sterile-distilled water. Since the use of sodium hypochlorite and bromine water did not prevent contamination, mercuric chloride was used as sterilizing agent throughout the experiment. Utmost care was taken, while using mercuric chloride and autoclaved hand gloves were employed, while sterilizing the explant.

Murashige and Skoog's (MS) medium (1962) containing 100 mg L^{-1} (wt vol^{-1}) silver nitrate, 5% (wt vol^{-1}) sucrose, fortified with cytokinins [N6- Benzyladenine (BA) (4.70-22.20 µm L^{-1}) or Kinetin (KN) (4.50-23.20 µm L^{-1})] and auxins [Naphthalene acetic acid (NAA) (0.56-2.77 µm L^{-1}), Indole-3-butyric acid (IBA) (0.59-2.56 µm L^{-1}) or Indole-3-acetic acid (IAA) (0.52-2.91 µm L^{-1})], 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 250 mL Erlenmeyer flasks (Borosil, India) and was autoclaved at 15 lb and 121^0C for 15 min. All the cultures were incubated at 252^0C at a relative humidity of 60-65% under 16 h photoperiod of 35-50 µmole m^{-2} s^{-1} irradiance provided by cool-white fluorescent tubes (Crompton Greaves, India).

To study the effect of different concentrations of sucrose and silver nitrate on the production of multiple shoots, the surface-sterilized nodal explants were cultured on MS medium supplemented with BA (16.80 µm L^{-1}), IBA (2.25 µm L^{-1}), sucrose (2-8%) and silver nitrate (0-200 mg L^{-1}). 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.

For multiplication of cultures, *in vitro* raised shoots were cut into pieces containing a single node along with dormant axillary buds and were taken in a sterilized petridish. The nodal portions 4-5 mm was excised 2 mm above the bud and 2 mm below the bud. Then the explants were transferred to 25 x150 mm culture tubes with 5 mL MS medium supplemented with silver nitrate (100 mg L^{-1}), BA (16.80 µm L^{-1}) and IBA (2.25 µm L^{-1}) for the induction of multiple shoots. Subsequently, subcultures were done at 25-days interval to study the effect of culture passages on the explant response for shoot induction and multiple shoot formation. Cultures were incubated at 27±2°C under the warm fluorescent light with intensity varied from 2000-3000 lux. The pH was adjusted to 5.7 prior to autoclaving.

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 & Cochran 1968).

RESULTS AND DISCUSSION

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 *V. negundo*, 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 I).

To study the effect of silver nitrate, in the presence of sucrose, on the induction of multiple shoots, the nodal explants were cultured on a medium supplemented with silver nitrate (0-200 mg L^{-1}), sucrose (2-8%) along with optimal concentrations of BA (16.80 µm L^{-1}) and IBA (2.25 µm L^{-1}). The conc. of 100 mg L^{-1} of silver nitrate was found most effective in the induction of multiple shoots, compared to other concentrations (Fig. 2).

The effect of culture passages was studied on multiple shoot induction in the subcultures of nodal segments of *in vitro* raised shoots (25 days-old) on MS medium supplemented with BA (16.80 µm L^{-1}) and IBA (2.25 µm L^{-1}) along with silver nitrate (100 mg L^{-1}). The highest response of nodal explants (98-100%) with a maximum average number of shoots (22) per explant was observed in the first five culture passages and then there was a gradual decline (Fig. 1A & B).

There have been several report of micropropagation with nodal and shoot tips of tropical medicinal plants in the juvenile phase of development (Kukreja *et al*., 1988). This signifies the potential for using juvenile material as a source of explants particularly for a beginner workering on tissue culture under tropical climate. Here the protocol is described for rapid and large-scale propagation of the woody aromatic and medicinal shrub *V. negundo* by *in*

vitro culture of nodal segments from mature plants. Here the different cytokinins were evaluated as supplements to Murashige and Skoog (MS) medium as the best medium (Boone *et al*., 1989). 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 the medicinal and aromatic plant species *Dictyospermum ovalifolium* (Thoyajaksha & Rai, 2001). The bud breaking and shoot induction in cultures of nodal explants indicate the function of cytokinins (Sahoo & Chand, 1998). In the present investigation, bud breaking and multiple shoot induction was increased in treatments of BA up to 16.80 μm. L^{-1} when supplemented with silver nitrate (100 mg. L^{-1}). A similar increase in the percentage of bud breaking and multiple shoot induction with increasing BA concentration up to 8.90 μm L^{-1} in *V. negundo*; however a declining trend was observed beyond this dosage (Sahoo & Chand, 1998). This increase in the bud breaking and multiple shoot induction in the present study may be attributed to the synergistic effect of silver nitrate and BA. A reduction in the number of shoots per explant was observed when the BA level increased beyond the optimal concentration (16.80 μm. L^{-1}). The percentage of explants responding for shoot induction (85-98%) and multiple shoot formation/explant (5-22) increased significantly on medium containing silver nitrate (100 mg. L^{-1}) along with optimum levels of BA (16.80 μm. L^{-1}) and IBA (0.59-2.56 μm. L^{-1}). The BA and IBA along with silver nitrate exhibited a synergistic effect on the percentage response of explants for shoot induction and multiple shoot formation. In each explant, 6-9 axillary buds were formed within 10-15 days after inoculation. Later, 25-30 days after inoculation, new shoots (10-25) were developed adjacent to these axillary buds. 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 IBA up to 2.25 μm. L^{-1}. Studies on *Gomphrena officinalis* (Mercier *et al*., 1992) and on *Rauvlfia serpentina* (Mathur *et al*., 1987) was also revealed the enhancing effect of medium fortified with cytokinins and auxins in shoot multiplication.

Again, 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 metal ions. 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 & Kersic, 1993). A mitochondrial enzyme, proline dehydrogenase, might play an important role during

Fig. 1A. The effect of culture passages on percentage of explant response for shoot induction

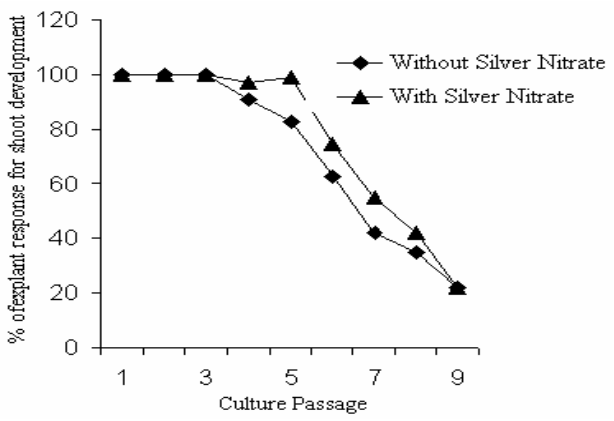
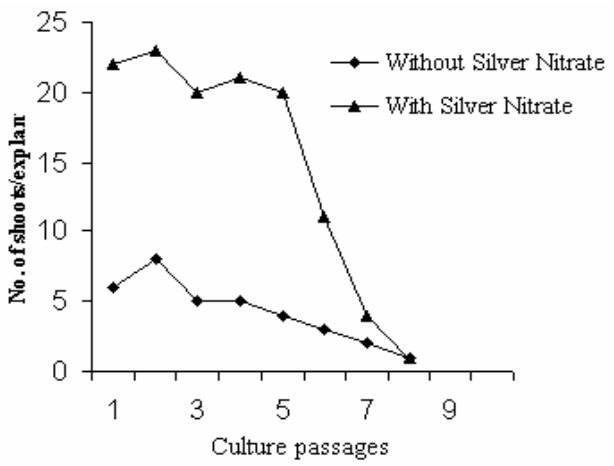


Fig. 1B. The effect of culture passages on multiple shoot formation



organogenesis (Kishor *et al*., 1999). A decline was observed in the proline accumulation in subsequent culture passages on the medium containing salt and noticed a negative correlation between proline accumulation and callus growth (Chandler *et al*., 1986). Silver nitrate, at the optimum concentration (100 mg. L^{-1}) 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 (Fig. 2). Increase in the induction of multiple shoots (22 shoots per explant) may be due to the synergistic effect of sucrose and salt. A gradual increase was observed in shoot multiplication and shoot elongation from the nodal explants of cashew nut (*Anacardium occidentale*) up to 4% sucrose and then there was a gradual decline (Boggetti *et al*., 1999). This study also led to the development of the C2D salts. Using these salts the rate of shoot multiplication was increased in comparison with salts of Murashige and Skoog that gave best results in previous experiments. The increase was from 40% to 350% depending on the variety.

In case of multiplication of shoots, similar

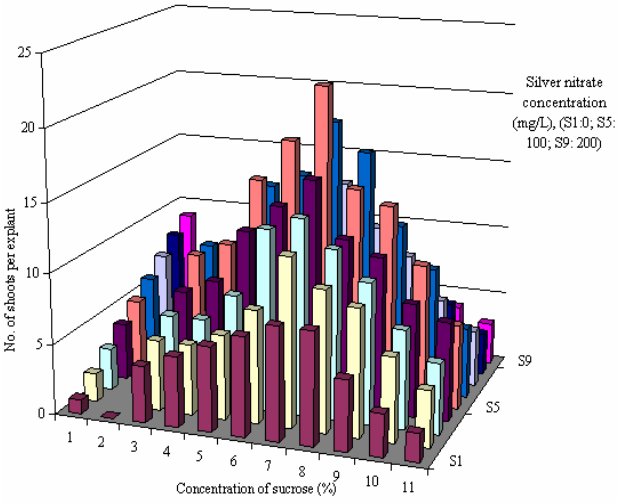
Table I. The effect of different concentrations of cytokinins, individually and in combination with auxins, on shoots induction and multiple shoot formation of nodal explants of *Vitex negundo* L

Cytokinin/ Auxin	Concentration ($\mu M.L^{-1}$)	% of explant response for Shoot induction	No. of Shoots/ explant	Shoot length (cm)
Basal				
KN	4.50	38.58±2.70	1.45±0.12	1.53±0.14
	9.40	49.29±2.51	1.98±0.14	1.67±0.12
	13.90	62.02±4.82	2.45±0.28	2.15±0.18
	17.40	67.78±2.72	2.14±0.21	2.43±0.15
	23.20	52.17±2.23	1.75±0.17	1.87±0.12
BA	4.70	65.25±3.35	1.98±0.23	2.98±0.19
	7.90	78.37±4.75	2.96±0.13	3.45±0.11
	13.30	85.41±4.33	4.95±0.21	3.63±0.12
	16.80	88.17±4.77	6.65±0.32	4.57±0.17
	22.20	78.01±3.71	1.95±0.11	2.59±0.03
KN+NAA	17.40+0.56	60.16±2.18	2.16±0.17	2.51±0.11
	17.40+1.57	65.22±2.71	2.97±0.25	2.68±0.18
	17.40+1.72	73.11±2.23	3.36±0.17	2.89±0.16
	17.40+2.25	71.56±2.24	2.96±0.23	3.89±0.14
	17.40+2.77	61.00±2.42	2.27±0.12	2.62±0.11
KN+IAA	17.40+0.52	57.56±3.21	2.18±0.16	2.49±0.12
	17.40+1.24	62.11±4.51	2.64±0.15	2.97±0.11
	17.40+1.75	70.90±2.56	3.07±0.27	3.32±0.10
	17.40+2.29	66.41±3.21	2.77±0.13	2.34±0.14
	17.40+2.91	57.86±2.45	2.41±0.18	1.91±0.05
KN+IBA	17.40+0.59	60.73±2.67	2.38±0.27	2.61±0.17
	17.40+0.97	68.12±3.57	2.89±0.21	2.75±0.12
	17.40+1.58	78.37±4.81	3.98±0.23	2.95±0.13
	17.40+1.98	71.29±2.52	3.33±0.15	4.09±0.05
	17.40+2.56	63.02±5.18	2.98±0.24	2.64±0.11
BA+NAA	16.80+0.56	75.45±2.87	3.96±0.21	3.05±0.17
	16.80+1.57	88.24±1.10	6.90±0.29	5.30±0.11
	16.80+1.72	93.23±2.81	9.19±0.14	4.88±0.15
	16.80+2.25	82.22±2.72	12.39±0.12	3.94±0.14
	16.80+2.77	73.14±2.16	5.01±0.11	3.24±0.15
BA+IAA	16.80+0.52	68.34±2.72	4.89±0.14	2.70±0.16
	16.80+1.24	78.50±2.81	6.47±0.28	2.80±0.17
	16.80+1.75	88.75±2.11	5.97±0.24	3.53±0.13
	16.80+2.29	84.25±2.34	4.87±0.17	2.36±0.10
	16.80+2.91	71.33±4.70	3.85±0.11	2.34±0.13
BA+IBA	16.80+0.59	88.88±2.92	5.53±0.27	4.08±0.11
	16.80+0.97	92.11±2.71	9.63±0.49	5.90±0.16
	16.80+1.58	96.16±2.12	12.98±0.67	6.83±0.17
	16.80+2.25	98.57±2.82	22.45±0.33	4.55±0.12
	16.80+2.56	85.11±2.13	7.07±0.63	3.94±0.17

Mean±SE

observations were made by Sahoo and Chand (1998) in *V. negundo* when subcultured on MS medium supplemented with BA [(4.40 µm. L^{-1}) (1.0 mg. L^{-1})] and GA3 [(1.15 µm. L^{-1}) (0.4 mg. L^{-1})] up to 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 salts and higher concentrations of sodium sulphate increased the necrosis (Chandler & 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 silver nitrate did not cause much difference in

Fig. 2. The effect of Silver nitrate, in the presence of sucrose, on multiple shoot induction from nodal explants of *Vitex negundo* L. on MS media supplemented with BA and IBA



the percentage of explant response for shoot induction (Fig. 1A); however, there was a 2-3 fold increase in the multiple shoot production (Fig. 1B). The results observed in culture passages, without salt, are in agreement with the observations of Sahoo and chand (1998).

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