# Phloroglucinol and silver nitrate enhances axillary shoot proliferation in nodal explants of *Vitex negundo* L. –an aromatic medicinal plant

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***Abstract***
A reliable method is described for rapid proliferation of axillary shoots from nodal segment of mature field-grown aromatic and medicinal plant *Vitex negundo* through in vitro culture. Of different concentrations of N6-benzyladenine (BA) evaluated as supplements to Murahige and Skoog (MS) medium, BA at 1mg/l was effective in enhancing bud break and shoot regeneration. Response of explants was also influenced by explanting seasons. Early bud break and enhancement of shoot regeneration could be achieved by supplementing phloroglucinol (PG) at 100mg/l and silver nitrate (20 mg/l) in addition to BA (1 mg/l). These compounds appear to have synergistic effects on enhancement of high frequency multiple shoot regeneration. The number of shoots obtained in the presence of $AgNO_3$ and PG was significantly higher (15.12±0.33) than other media tested. The efficacy of culture establishment with high percentage of responding explants is depending on seasonal factors in addition to culture conditions. Among the different concentrations of IBA (0.1, 0.5 and 1.0 mg/l) tested, 0.5mg/l IBA was more effective for inducing roots within two weeks of culture and 85% of the microshoots produced long healthy root system. *Ex vitro* rooting of micropropagated shoots was achieved by treating the basal end of the microshoots in IBA solution (4000 ppm) followed by planting in plastic pots filled with a mixture of soil, sand and vermicompost (6:2:1). Rooted microshoots were successfully established in the field with 80 percent survival rate after three months.
***Keywords:*** *Vitex negundo*; micropropagation; shoot regeneration; *Ex vitro* rooting

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## INTRODUCTION

*Vitex negundo* L. (Verbenaceae) is an important medicinal plant in India and widely used in Indian system of medicine. It is a large woody aromatic and medicinal shrub or sometimes a small tree distributed in several parts of India (Anonymous, 1976). In recent years, unrestricted removal and over exploitation of this medicinal plant for the preparation of various formulations of medicines leads to drastic reduction of this important genetic resource in India. The ever-increasing demand for *V. negundo* is also due to limited cultivation and insufficient attempts for large-scale establishment. Presently, the wild genetic stock of *V. negundo* has been depleted heavily due to various biotic and abiotic stresses (Sahoo and Chand, 1998). Conventional method of propagation of *V. negundo* and several other medicinal plants through vegetative cuttings is unfortunately slow and needs large number of stem segments from the mother plants, leading to more destruction of available genetic stocks (Sharma *et al*., 1991). The efficacy of rooting is strictly age dependent besides several other limiting factors. Alternatively, seed propagation of *V. negundo* is possible only with very poor rate of germination. Owing to these reasons, it is very important to protect and restore *V. negundo* by developing a viable protocol for its large-scale micropropagation method and also to supply adequate quantity of elite clones for establishment in the field.

Ever increasing interest on *in vitro* culture techniques has been applied not only for multiplication of several rare species of great importance but also for

cloning of elite types of plants on a larger scale. *In vitro* techniques are effectively utilized for germplasm conservation of rare, endangered aromatic and medicinal plants (Arora and Bhojwani 1989; Sudha and Seeni, 1994). Considering the medicinal and aromatic values of *V. negundo*, attempt was made to standardize micropropagation technique for cloning of elite trees (Sahoo and Chand, 1998) no further reports are available on micropropagation of *V. negundo*. In this paper, we describe a simple and reliable protocol for multiplication of this pharmaceutically important plant through high-frequency axillary shoot proliferation using silver nitrate and phloroglucinol. The importance of present finding and its possible application in the context of conservation and multiplication of this plant are discussed.

## MATERILAS AND METHODS

**Plant material:** Actively growing young shoots of *V. negundo* L. with four to five nodes were collected in different months from the mature field-grown plants maintained in the botanical garden of Sri Paramakalyani Centre for Environmental Sciences, Manonmaniam Sundaranar University. Nodal segments approximately measuring about 1.5 cm were cut from the shoots after removing the leaves. The nodal segments were rinsed with water for 10-15 min and then disinfected using 0.1% (w/v) mercuric chloride (BDH, India) for 7-10 min. Thereafter, the segments were washed 5-7 times with sterile distilled water. Nodal explants were trimmed using a sterile surgical blade under a mixture of ascorbic and citric acid solution (0.1% each) and blotted on sterile filter paper before implanting on the media.

**Culture medium and conditions:** The culture medium used for the present work includes Murashige and Skoog's (1962) medium (MS) supplemented with sucrose (3%). The medium was further augmented with BA of different concentrations (0, 0.1, 0.5, 1, 5 and 10 mg/l). In addition, different concentrations of $AgNO_3$ (2.5, 5, 10, 15, 20, 25, 50mg/l) and PG (25, 50, 75, 100, 150, 200 mg/l) were individually tested for optimizing the use of these compounds along with BA. A separate experiment was performed using selected concentration of silver nitrate (20 mg/l) and phloroglucinol (100 mg/l) either alone or in combination with BA for enhancing the shoot proliferation. The pH of the medium was adjusted to 5.8 before gelling with 0.8% agar (Hi-media, India). All the chemicals used in the present study were of analytical grade (British Drug House, Sigma, Merck, and Hi-media). Molten medium was dispensed into 200 ml screw-capped glass jars or 150 ml Erlenmeyer flasks (Borosil, India) and capped with cotton plugs before sterilization at 121°C for 20 min explants were implanted vertically on the culture medium and incubated at 25±1°C under 16hrs photoperiod. The number of explants cultured in each treatment varied from 40-60 depending upon the experiments.

**Multiplication, *ex vitro* rooting and acclimatization:** Primary cultures with single and multiple shoots regenerated from the original nodal explants were carefully taken out from the culture vessels and sectioned into nodal segments. Nodal segments were once again cultured on MS medium fortified with BA (1 mg/l), $AgNO_3$ (20 mg/l) and PG (100 mg/l) for further multiplication. Multiplication of shoots was carried out by repeated harvest of microshoots followed by sub-culturing of nodal explants on medium supplemented with BA (1 mg/l), $AgNO_3$ (20 mg/l) and PG (100 mg/l). For *in vitro* rooting, healthy microshoots regenerated from the above experiments were subjected to rooting on half-strength MS medium fortified with different concentrations of indole butyric acid (IBA) (0.1, 0.5 and 1.0 mg/l). Microshoots measuring the height of 4-5 cm with 2-3 pairs of leaves were cultured on different concentrations of IBA and maintained under the cultural conditions as described earlier. For *ex vitro* rooting, long healthy microshoots were trimmed at the basal end to expose the fresh tissues for facilitating the absorption and treated with 4000 ppm IBA solution and planted in the plastic pots containing soil, sand and vermicompost (6:2:1).

Plantlets with well developed root system were recovered from the culture vessels and gently rinsed to remove the adhering media before transfer into plastic pots containing a mixture of soil, sand and vermicompost for acclimatization. Plantlets were initially grown under diffused natural light in a small polythene tunnel with frequent misting to maintain the humidity. Regenerated plants were nourished with diluted macro and micro nutrient of MS medium (1/8) for better growth and development. After 45 days, 75% of the acclimatized plants with well developed root and shoot system were transferred to natural conditions under a shade. These plants were subsequently established in the field by providing frequent watering with organic compost.

## RESULTS

**Seasonal effects on bud break and shoot regeneration:** Recovery of aseptic explants and responses with regard to bud break and shoot development varied in different seasons. Use of $AgNO_3$ and PG at 20 and 100mg/l respectively was found to influence maximum shoot proliferation (Fig. 1 A,B). Nodal explants sourced from actively growing shoots (Fig. 2 A) and cultured during March-May, recorded maximum recovery of aseptic culture (60%). Shoots established during these months responded well with maximum bud break (95%) and also with more number of shoots per explant (7.29±0.28). Decline in bud break (55.2%) and shoot proliferation (3.37±0.37) was observed during June-August with poor recovery of explants (35%). Explants established during September-November exhibited poor bud break (26%) with less number of shoots per explant (2.2±0.21). Response of explants was improved during December-February (52%) with better shoot proliferation (Table 1). Recovered nodal explants often responded well during initiation of nodal cultures. However, few days after initiation, tissue browning followed by death of explants was commonly noticed in many cultures throughout the year. Most of the explants developed callus or abscission like outgrowth followed by poor sprouting and shoot proliferation in absence of PG and $AgNO_3$ (Fig. 2 B,C). In our study, MS medium fortified with $AgNO_3$ (20 mg/l) effectively controlled abscission-like development on the explants and enhanced shoot regeneration (Fig. 2 D,E).

**Shoot regeneration and multiplication:** Explants cultured on MS basal medium and also with lower concentrations of BA (0.1 and 0.5 mg/l) exhibited poor shoot length ranging from 14.2-19mm with 1-2 pairs of leaves (Table 2). Increase in concentration of BA (1 mg/l) induced longest shoots (28.1 mm) with 3-4 pairs of leaves over other concentrations of BA tested (Fig. 2 F). Therefore, BA (1 mg/l) was found to be the best and optimum concentration to induce healthy microshoots. An average of 10 shoots per explant was induced at this concentration. Increase in concentration of BA to 5 and 10 mg/l induced more number of shoots. However, these shoots were found weaker with shorter internodes. The leaves produced by these shoots often found very smaller compared with the shoots obtained at lower concentrations of BA. Higher concentrations of BA (5 and 10 mg/l) developed compact callus at the basal end of the explant.

Response of explants varied from 60-95% depending on the presence of BA, $AgNO_3$ and PG in the medium (Table 3). Explants cultured on medium fortified with BA (1 mg/l) or $AgNO_3$ (20 mg/l) did not show any noticeable differences with respect to shoot

**Table 1.** Effect of explanting season on establishment of nodal culture of *Vitex negundo* L. on MS medium fortified with BA (1 mg/l). Mean value (± SE) were collected after 45 days from four replications, each replication with 30 explants.

| Period of culture | Mar-May | Jun-Aug | Sep-Nov | Dec-Feb |
|---|---|---|---|---|
| Recovery | 60% | 35% | 43% | 52% |
| Bud break | 95.29 ± 3.29 | 55.27 ± 2.53 | 26.00 ± 0.42 | 49.32 ± 0.21 |
| Shoots/explant | 7.29 ± 0.28 | 03.37 ± 0.37 | 02.20 ± 0.21 | 04.23 ± 0.32 |

**Table 2.** Response of nodal explants of *Vitex negundo* L in different concentrations of BA. Mean value (±SE) were collected from four replications, each with 25 explants. Duration of Culture 45 days.

| BAP (mg/l) | Shoot development(%) | Shoot number/ explant | Shoot length (mm) |
|---|---|---|---|
| Control | 13.36 ± 1.26 | 1.00 ± 0.00 | 14.25 ± 2.60 |
| 0.1 | 20.23 ± 1.32 | 1.32 ± 0.23 | 10.62 ± 1.72 |
| 0.5 | 24.26 ± 1.43 | 2.25 ± 0.32 | 18.50 ± 1.70 |
| 1.0 | 58.36 ± 1.26 | 2.25 ± 0.32 | 28.10 ± 1.70 |
| 5.0 | 64.26 ± 1.62 | 10.42 ± 0.62 | 19.25 ± 2.20 |
| 10 | 40.16 ± 2.16 | 12.12 ± 0.33 | 19.00 ± 2.70 |

**Table 3.** Effect of PG and $AgNO_3$ on shoot proliferation in nodal explants of *Vitex negundo*. Duration of Culture 60 days. Values (± SE) are mean of 30 proliferating cultures.

| Treatments | Shoot Development (%) | Shoot number/explant | Shoot length (mm) |
|---|---|---|---|
| Control | 14.16 ± 1.12 | 1.00 ± 0.00 | 16.22 ± 2.10 |
| BAP (1 mg/l) | 60.32 ± 1.26 | 2.32 ± 0.23 | 19.26 ± 1.62 |
| PG (100 mg/l) | 32.26 ± 1.42 | 2.25 ± 0.24 | 18.50 ± 1.70 |
| $AgNO_3$ (20 mg/l) | 38.36 ± 1.26 | 3.25 ± 0.32 | 18.50 ± 1.70 |
| BAP (1 mg/l) + PG (100 mg/l) + $AgNO_3$ (20 mg/l) | 95.16 ± 1.16 | 15.12 ± 0.33 | 27.80 ± 1.72 |

length. Addition of PG (100 mg/l) alone in the medium was found to have positive response as evidenced by increase in shoot length (18.50±1.70) over control (16.22±2.10). However, combination of BA, $AgNO_3$ and PG was found to be the best treatment to induce highest number of shoots per explant (15.12±0.33). The present study indicated that though BA at 1mg/l induced less number of shoots per explant (2.32±0.23), addition of other adjuvants such as $AgNO_3$ and PG along with BA enhanced bud break within a week of initial culture and significantly enhanced the number of shoots per explants (15.12±0.33) within 60 days (Table 3 and Fig. 2 G, H and I).

**Root induction and acclimatization:** Shoots cultured on MS medium fortified with different concentrations of IBA (0.1, 0.5 and 1.0 mg/l) responded well with varying percentage of rooting. Among the three different concentrations tested, 0.5 mg/l IBA was most effective for induction of roots. About 85% of the excised shoots developed healthy roots within two weeks of culture and no callus formation was observed from the proximal end of the shoots (Fig. 2 J). The rooting percent was reduced to 65 and 42% at 0.1 and 1.0 mg/l IBA respectively. Compact brown callus development was frequently seen in many shoots when 1.0 mg/l IBA was used in the medium. *Ex vitro* rooting was attempted using 120 microshoots by exposing the basal end of the shoots in a sterile IBA solution (4000 ppm) for few seconds followed by planting on a substrate as described earlier. About 75% of the shoots produced healthy root system and exhibited good shoot growth. Rooted microshoots could be hardened within 45 days (Fig. 2 K, L) and subsequently established in the field.

## DISCUSSION

*In vitro* responses of nodal explants of *Vitex negundo*

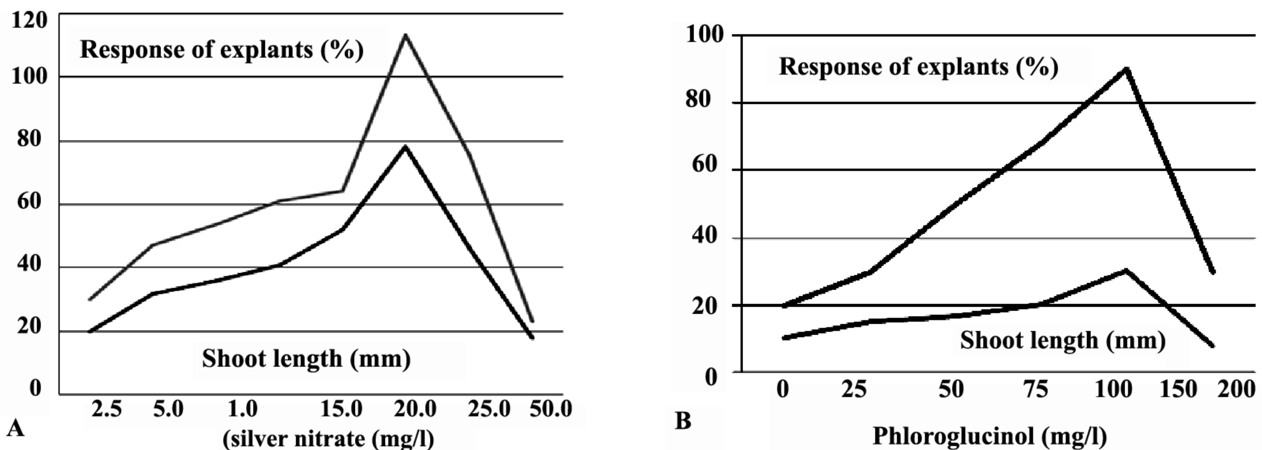


**Figure 1.** A and B: Effect of different concentrations of $AgNO_3$ and PG on bud break and shoot regeneration in nodal explants of *V. negundo* cultured on MS medium for 45 days.

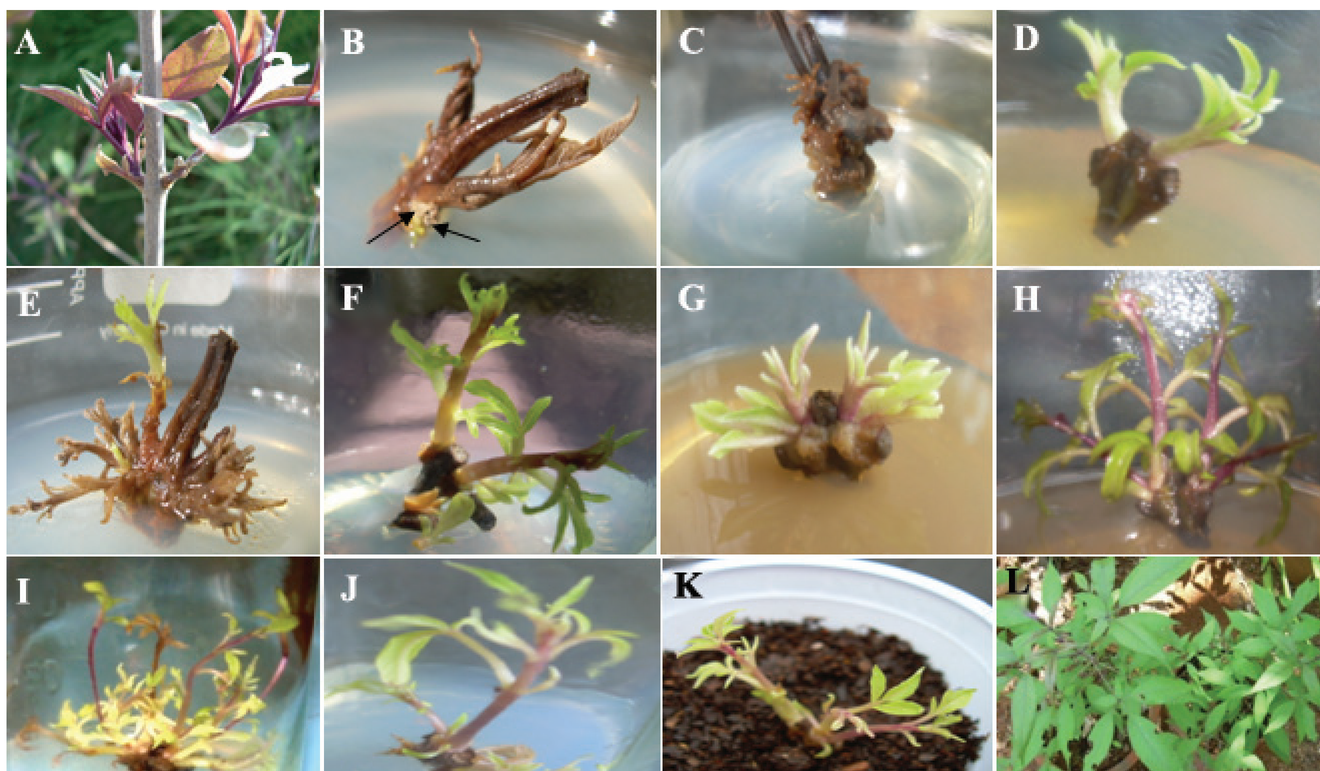


**Figure 2.** Micropropagation of *Vitex negundo*: A: actively growing shoots from the stock plant; B: intitiation of abscission (shown with arrow mark) in nodal explants; C: browning and death of explant due to abscission; D: axillary shoot proliferation from nodal explant during initial culture; E: death of multiple shoots after prolonged culture period; F: shoot regeneration using BA(1 mg/l); G and H: enhanced bud break and shoot regeneration using phloroglucinol (100 mg/l) and silver nitrate (20 mg/l); I: high frequency multiple shoot regeneration using a combination of BA (1 mg/l), phloroglucinol (100 mg/l) and silver nitrate (20 mg/l); J: root induction on MS medium supplemented with IBA (1 mg/l); K: initial hardening of plantlet; L: fully hardened plants for field planting.

was primarily influenced by changes in vegetative growth pattern of mature trees. Establishment of aseptic culture become easy during March-May and December-February as 70-80% of the explants responded well within two weeks of initial culture and grew faster during subsequent culture period. The better response of nodal explants under *in vitro* condition during these periods was comparable with the active shoot development in the field-grown tree. Cultures initiated during June-November did not proliferate into shoots despite using the higher concentration of BA in the medium, possibly due to dormancy of axillary buds prevailed during these months. It was generally noticed that the vegetative growth of *V. negundo* was very poor during June-November and there was no rejuvenation of vegetative growth and development in spite of frequent watering. Influence of explanting season on bud break and shoot proliferation has previously been reported in *V. negundo* (Sahoo and Chand, 1998). They reported that the season of explanting was crucial for establishment of cultures as experienced in the present study. Similar results were reported in other medicinal plants such as *Eucalyptus* (Das and Mitra, 1990) and *Ocimum* species (Ahuja *et al*., 1982; Pattnaik and Chand, 1996).

Determination of optimal concentrations of $AgNO_3$ for use in micropropagation of several plant species has been reported. For instance, $AgNO_3$ at 20 mg/l was found effective in nodal culture of *Theobroma cocoa* (Mallika *et al*., 1996), *Coffea arabica* (Ganesh, 2000; Ganesh and Sreenath, 2008) and *Vanilla planifolia* (Sankar *et al*., 2008). Similarly, beneficial effect of PG was determined at the concentrations ranging from 80-200mg/l in a variety of plant species. In *Coffea,* PG at 200mg/l was reported as an optimum concentration for enhancing shoot proliferation (Ganesh and Sreenath, 2008). In *Minuartia valentia*, PG at 80 mg/l was found effective for regeneration of shoots from nodal segments (Ibanez and Amo-Marco, 1998). More recently, micropropagation was demonstrated by

inducing early bud break and shoot regeneration using PG (100 mg/l) in nodal explants of *Mallus domstica* (Rustaei *et al*., 2009). In our study, the effective concentrations of $AgNO_3$ and PG were 20 and 100 mg/l respectively for enhancing bud break and shoot regeneration in *V. negundo*.

During initial culture, many of the explants showed abscission-like development in the nodal explants and often limited shoot proliferation. A similar observation was reported earlier in many plant species due to accumulation of ethylene produced by wounded explants (Chi and Pua 1989). Silver nitrate is reported to be one of the potent inhibitors of ethylene. It inhibits ethylene activity through Ag ions by reducing the receptor capacity to bind ethylene (Yang, 1985). Silver nitrate has several useful properties such as promoting root induction in apple, chicory plants and *Decalepsis hamiltonii* (Ma *et al*., 1998; Bais *et al*., 2000), maturation of somatic embryos of an important medicinal plants *Andrographis paniculata* (Martin 2004) and enhancement of shoot proliferation in nodal culture of *Vanilla planifolia* (Ganesh *et al*., 1996; Giridhar *et al.,* 2001; Ayyappan, 1990). In our study, MS medium fortified with silver nitrate significantly reduced the abscission-like development that is generally caused due to production of ethylene by injured explants.

High frequency shoot proliferation was significantly reduced when higher concentrations of BA were supplemented in the media. The shoots were highly condensed with very shorter internodes and also with reduced leaf size as reported earlier by several workers in various plant species such as *Pogostemon cablin* (Kukreja *et al*., 1990), *Withania somnifera* (Sen and Sharma 1991) and *Kaempferia galango* (Vincent *et al*., 1992). Sahoo and Chand (1998) tested different cytokinins such as KN, BA and TDZ for regeneration of shoots from nodal explants of *V. negundo*. They found that BA at 2.0 mg/l was most effective in inducing bud break and multiple shoot formation. It was also reported that use of GA in combination with BA have synergistic effect on multiple shoot formation and 4-5 shoots per explant were induced from the nodal explants of mature field-grown plants. In general, use of GA is not preferable for long term culture as GA inhibits rooting in the subsequent phases of micropropagation and hence GA was not used in our study. In the present study, BA at 1 mg/l was found optimum for induction of healthy microshoots and increase in concentration of BA beyond this level causing callus development at the basal end of the explant, leading to reduced shoot development. Stimulating effect of BA on bud break and shoot formation has been well studied in several other medicinal and aromatic plants including *Chlorophyllum borivilianum* (Purohit *et al*., 1994), *Ocimum* spp. (Puttnaik and Chand, 1996), *Piper* spp. (Bhat *et al*., 1995). *Pogostemon cablin* (Kukreja *et al*., 1990) and *Withania somnifera* (Sen and Sharma 1991).

Number of shoots obtained in every cycle of micropropagation is crucial for large-scale propagation of plant species. In the present study, PG and $AgNO_3$ acted synergistically with BA in inducing early bud break and high frequency shoot proliferation (15 shoots per explant). Further, it was observed that omission of any one of these compounds did not give satisfactory results with respect to shoot regeneration. Phloroglucinol is a phenolic compound predominantly found in xylem sap of apple and is known to promote growth and development in a number of plant species such as *Chinchona ledgeriana* (Hunter, 1979), rasaceous fruit trees (Singha 1980; Zimmerman and Broome 1981) and Cocoa (Mallika *et al*., 1996). In our study, the results on the usefulness of PG on shoot proliferation are comparable with the previous reports in cocoa *(*Mallika *et al*., 1996). *In vitro* shoot proliferation has previously been reported in a wide range of medicinal plants by combining different auxins and cytokinins such as BA+IAA for *Adhatoda beddomei* (Sudha and Seeni, 1994), BA+IBA for *Rheum emodi* (Lal and Ahuja 1989), BA+NAA for *Gomphrena officinalis* (Mercier *et al*., 1992) and BA+KN for *Kaempferia galanga* (Vincent *et al*., 1992). However, usefulness of adjuvants such as $AgNO_3$ and PG has been reported only in a limited species. The present study demonstrated that PG and $AgNO_3$ are potent compounds for regeneration of high frequency shoots in *V. negundo*. Our study also indicated the possibility of utilizing these compounds in micropropagation of other plant species.

Sahoo and Chand (1998) induced roots from the regenerated shoots of *V. negundo* by using a combination of two auxins namely IBA (1 mg/l) and IAA (1 mg/l) followed by root elongation on hormone-free half-strength MS medium. By contrast, in the present study, we have demonstrated root induction and elongation simultaneously using a single auxin (IBA) and thereby reduced the time required for healthy root induction. The present study also demonstrated a reliable *ex vitro* rooting for production of plantlets. *Ex vitro* rooting is very popular and advantageous over *in vitro* rooting as this method helps in rooting and acclimatization simultaneously. In our study, when

microshoots of *V. negundo* were experimented for *ex vitro* rooting, high percentage (85%) of rooting was achieved. *Ex vitro* rooting has been demonstrated in a wide range of plant species such as *Rhododendron* (Kyte and Briggs 1979, McCown and Lloyd 1983; Ettinger and Preece 1985), *Coffea arabica* (Rajasekaran and Mohankumar 1993) and several other species. To reduce *in vitro* manipulations and also to minimize the cost of production, *ex vitro* rooting is very often practiced for commercial production of several horticultural and forest tree species. In the present study, it is demonstrated that *ex vitro* rooting is effective and suitable for micropropagation of *V. negundo*. The rooted shoots planted in the semi sterile soil mixture containing vermin compost grew normally and produced 3-4 pairs of fresh leaves. During initial hardening, the plantlets exhibited with short internodes and smaller leaves. The plantlets grew well and attained healthy conditions for field planting when these plantlets were transferred to earthen pots containing soil mixture. Sahoo and Chand (1998) evaluated different combinations of substrate (vermiculite, vermin-compost, soilrite mix, garden soil) for acclimatization of micropropagated *Vitex negundo*. They reported that vermicompost supported maximum survival of micropropagated plants with enhanced shoot and root development. In our study, 75% of micropropagated plantlets exhibited good vegetative growth and attained requisite stage for field planting.

The present work demonstrated the possibility of micropropagation of *Vitex negundo* through *in vitro* methods. Considering the importance of medicinal and other aromatic properties of *V. negundo*, micropropagation protocols can be effectively used in this species for various applications such as *in vitro* conservation, cryopreservation, large- scale multiplication and genetic transformation. Besides, the present study also revealed the usefulness of silver nitrate and phloroglucinol for enhancing shoot regeneration in one of the important medicinal and aromatic plants.

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