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IN VITRO REGENERATION FOR MASS PROPAGATION IN COMMERCIAL SCALE OF MEDICINAL PLANT *VITEX NIGUNDO* L.

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Abstract

Context: It is necessary to focus on the importance of adopting micropropagation technique for mass propagation of the plantlets in commercial scale as well as conservation and distribution of germplasm.

Objective: The present investigation has been designed with a view to establishing protocol of *in vitro* regeneration of medicinal plant species i,e., *Vitex nigundo* L (Verbenaceae).

Materials and Methods: Shoot tips and nodal segments were used for multiple shoot induction. All explants were cultured on MS medium supplemented with various plant growth regulators. $HgCl_2$ was used as surface sterilizing agent. For *in vitro* rooting, individual shoots (3-4 cm) were cut from the proliferated shoot cultures and implanted on half and full strength of MS with different concentrations and combinations of NAA and IAA. The cultures were incubated for 16 h photoperiod at 25 ± 2ºC under a fluorescent light. Visual observation of culture was made every week. Data on shoot induction and proliferation and root induction were recorded after three weeks of inoculation and used for calculation. For each treatment 15 explants were used and all the treatments were repeated thrice. Established plantlets were transplanted in earthen pots under natural conditions and the survival rate was recorded.

Results: The most effective surface sterilization treatment has been found 0.1 % $HgCl_2$ for 7 minutes. Highest number of shoot was observed in MS medium supplemented with 3.0 mg/ BAP. It was rooted well in full MS containing 2.0 mg/l IAA. The survival rate was 85 % and propagated plantlets were successfully acclimatized in soil.

Conclusion: It was observed that shoot tips are more responsive for micropropagation of *Vitex nigundo* L . Thus the fruitful utilization of rapid clonal propagation, germplasm conservation and distribution of *Vitex nigundo,* important medicinal plant of Bangladesh, is possible.

Keywords: *Vitex nigundo,* Medicinal plant, Shoot induction, Micropropagation, Regeneration.

Introduction

In spite of being a rich source of medicinal plant, Bangladesh depends on import for raw materials of pharmaceuticals. *Vitex nigundo* L. is commonly known as 'Nishinda' belongs to the family Verbenaceae, a woody aromatic and medicinal shrub (Sahoo and Chand 1998). This plant grows gregariously in wastelands and is also planted as a hedge-plant. It is an erect, 2–5 m in height, slender tree with quadrangular branchlets distributed throughout Bangladesh (Ghani 2003), India, Ceylon, Afghanistan, Philippine Islands and Tropical Africa, Madagascar and China (Bansod and Harle 2009). The leaves have five leaflets in a palmately arrangement, which are lanceolate, 4–10 cm long, hairy beneath and pointed at both ends. The bluish purple flowers are numerous. The fruit is succulent, black when ripe, rounded and about 4 mm in diameter (Mandak *et al.* 1976, Singh *et al.* 1999).

Phytochemical investigation of this plant indicated the presence of monoterpenes agnuside, flavonoids-casticin, chryso-splenol and vitexin, flavonoids (vitexicarpin) also, 5,3 -dihydroxy-3,6,7,4 -tetramethoxyllavone and hydroxy-3,6,7,3 ,4 -penta methoxy flavone from the leaves (Achari *et al.* 1984). Seeds of *V. negundo* agorded a new lignin characterized as 6-hydroxy-4-4-hydroxy-3-methoxyphenyl), 3-hydroxymethyl-7-methoxy-3,4-dihydro-2-naphthaldehyde by spectroscopic methods and triterpenoids (betulinic acid and ursolic acid), lignans (negundins, vitedonin), alkaloid (vitrafalal) and diterpene (vitedoin) investigated (Chawla *et al.* 1992).

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The leaves *V. negundo* are antibacterial, antitumor, astringent, febrifuge, sedative, tonic and vermifuge (Chopra *et al.* 1986). Extracts of the leaves have insecticidal activity (Duke and Ayensu 1985). The fresh leaves are burnt with grass as a fumigant against mosquitoes (Bown 1995). The fruit is also used in the treatment of angina, colds, coughs, rheumatic difficulties etc (Duke and Ayensu 1985). The root is expectorant, febrifuge and tonic (Chopra *et al.* 1986). It is used in the treatment of colds and rheumatic ailments (Duke and Ayensu 1985).

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 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 (Sudha and Seeni 1994). Considering the medicinal and aromatic values of *V. negundo*, very few attempts were made to standardize micropropagation technique for cloning this plant. In this paper, we describe a simple and reliable protocol for multiplication of this pharmaceutically important plant through high-frequency axillary shoot proliferation.

Materials and Methods

V. nigudo plants collected from the wild population of Jhenidah, Bangldesh were used as the explant sources for micropropagation. Different kinds of plant growth regulators i.e., BAP (6-benzyl amino purine), zeatin of cytokinin group and NAA (Napthalene acetic acid), IAA (Indole-3 Acetic Acid), 2,4 D (2,4-dichlorophenoxy acetic acid) of auxin group were used for this experiment. $HgCl_2$ was used as surface sterilizing agent.

For plant growth MS medium containing nutrient basal salts were used which content macro, micro elements and vitamins. The explants were washed thoroughly under running tap water, presoaked in liquid detergent for about 30 min. They were then surface sterilized with 0.1% (w/v) mercuric chloride for 7 min, followed by five rinses with sterile distilled water inside a clean ench. The surface sterilized explants were sized to 3-4 cm in length containing a single node with an axillary bud or a shoot tip with an apical bud. The explants were placed vertically on the culture medium.

For shoot induction agar-gelled MS (Murashige and Skoog 1962) basal media supplemented with BAP (6-benzyl amino purine), zeatin were prepared. The new shoots induced from the *in vitro* cultures were further used as explants for adventitious shoot regeneration. For *in vitro* rooting, individual shoots were cut (3-4 cm) from the proliferated shoot cultures and implanted on half and full strength of MS with different concentrations and combinations of NAA and IAA. All media were supplemented with 3% sucrose and gelled with 1% agar. Prepared media were dispensed into test tube (150×25 mm). The pH of the media was adjusted to 5.8 before autoclaving at 121°C for 20 min. The cultures were incubated for 16 h photoperiod at 25 ± 2°C under a fluorescent light.

Visual observation of culture was made every week. Data on shoot induction and proliferation and root induction were recorded after three weeks of inoculation and used for calculation. For each treatment 15 explants were used and all the treatments were repeated thrice. The healthy plantlets were taken out from the culture tubes, washed to make free from agar gel with running tap water and transplanted to plastic pots containing soil, sand and compost (1:1:1) for hardening. Established plantlets were transplanted in earthen pots under natural conditions and the survival rate was recorded.

Results

In the present investigation it was observed that 90% explants were effective to make contamination free with no tissue damage when treated with 0.1% $HgCl_2$ solution for 7 min. When BAP was used in different concentrations, the highest mean numbers of shoots per explant was found from shoot tips and nodal segments in media having 3.0 mg/l BAP and highest mean length of the shoot was also recorded in these media (Table 1). It was rooted well in full MS containing 2.0 mg/l IAA. The highest mean number of shoots per explants was produced in media having 3.0 mg/l BAP + 0.1 mg/l NAA. Shoot tips were found better than nodal segments for high frequency of shoot multiplication in *V. nigundo*. It was observed that when shoot tips and nodal segments were cultured on MS medium supplemented with BAP and Zeatin singly in different concentrations, BAP was proved better than Zeatin for shoot multiplication in the present study.

It was observed that IAA was superior to other auxin (NAA), when used singly for rooting. Among the different concentrations tested in both half and full strength of MS medium supplemented with 2.0 mg/l IAA was found to be the best level for root induction and growth of roots. In the present study, it was observed that using different concentration of NAA and IAA on full strength of MS medium is better than that of half strength of MS medium for root induction. In the present investigation, maximum root induction was found in MS medium supplemented with 2.0 mg/l IAA (Table 2). Shoot regenerated from shoot tips and nodal segments explants were rooted to develop complete plantlets (Fig. 1 A-F).

The plantlets developed from different in *in vitro* cultures were successfully established in soil. In the present investigation, the survival of acclimatized plant is 85%, the highest mean numbers of shoots per explant were found from shoot tips and nodal segments in media having 3.0 mg/l BAP and highest mean length of the shoot was also recorded in these media. It was rooted well in full MS containing 2.0 mg/l IAA. So, for high efficiency micropropagation, it is recommended that nodal explants can be cultured for 6-7 weeks in 3.0 mg/l BAP.

Table 1. Effect of cytokinins for micropropagation of *Vitex nigundo using* shoot tips and nodal segments isolated from field grown plant for multiple shoot development.

Treatment(mg/l)		Response of explants (%)	Average no. of shoot / culture	Average length of shoot (cm)	Response of explants (%)	Average no. of shoot / culture	Average length of shoot (cm)
		Shoot tips of *Vitex nigundo*			Nodal segments of *Vitex nigundo*		
MS (Control)		25	3	2	25	3	2
MS + BAP	0.5	37.5	4	2.5	50	3	3
	1.0	50	5	3.1	87.5	4	3.2
	2.0	75	6	3.5	87.5	3	4.2
	3.0	87.5	7	4.4	87.5	6	4.3
	4.0	75	4	3.9	75	5	4.1
MS + BAP + NAA	1.0+0.1	62.5	5	2.7	50	5	2.5
	1.0+0.5	62.5	5	3	62.5	4	3.0
	2.0+0.1	75	6	3.9	87.5	5	4.0
	2.0+0.5	75	5	4.2	75	5	4.1
MS +BAP + IAA	1.0+0.1	50	4	3	50	4	3
	1.0+0.5	62.5	3	3	62.5	5	3
	2.0+0.1	75	5	3.7	62.5	5	3.7
	2.0+0.5	75	6	4	75	6	4
MS + BAP + 2,4-D	1.0+0.1	75	4	2	75	4	2
	1.0+0.5	62.5	5	2.6	87.5	5	2.6
	2.0+0.1	87.5	6	4.0	87.5	6	4
	2.0+0.5	75	6	4.0	75	5	4

Table 2. Effect of different concentration of NAA and IAA in full and half strength of MS medium for root induction of the elongated micro shoots of *Vitex nigundo.*

Treatment (mg/l)		Days to response	Response of explants (%)	No. of root /culture	Average length of root (cm)
MS+ NAA	0.1	11	62.5	2	1.4
	0.5	9	75.0	3	1.8
	1.0	10	75.0	4	2.1
	2.0	12	87.5	4	1.9
MS +IAA	0.1	14	75.0	3	2.0
	0.5	11	87.5	3	1.7
	1.0	10	75.0	4	2.1
	2.0	9	87.5	6	2.5
1/2MS+ NAA	0.1	10	75.0	3	1.7
	0.5	9	62.5	2	1.9
	1.0	12	75.0	5	2.0
	2.0	14	75.0	4	2.2
1/2MS+ IAA	0.1	16	87.5	2	1.6
	0.5	16	75.0	1	1.9
	1.0	13	87.5	3	1.4
	2.0	14	87.5	4	2.0

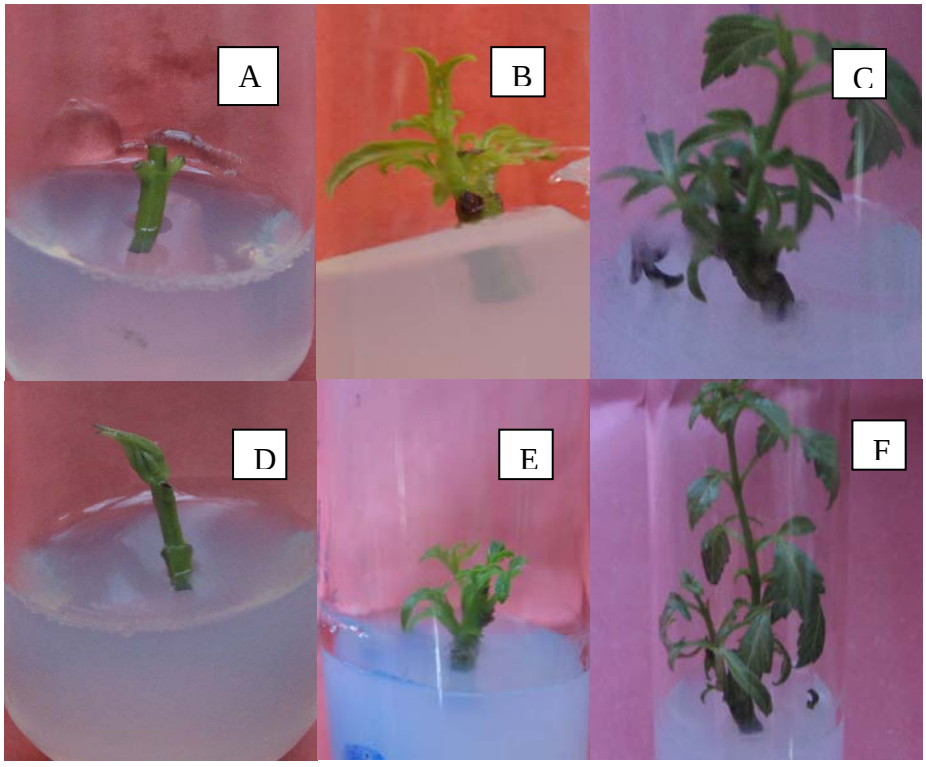

Fig. 1. Regeneration of plantlets *in vitro* from nodal segments and shoot tips of *Vitex gundo*. (A) Inoculation of nodal segment explants on regeneration medium at first day;(B) Shoot regeneration from nodal segment explants; (C) Shoot development after 6 weeks; (D) Inoculation of shoot tips explants on regeneration medium at first day. (E) Shoot regeneration from nodal segment explants; (F) Shoot development after 6 weeks.

Discussion

Different concentrations of cytokinin alone or in combination of auxin were used to see the response of shoot multiplication from shoot tip and nodal segment explants. Cytokinins have been shown by many workers to release lateral buds from apical dominance and also from inhibition by auxins. The shoot development as well as the rooting of regenerated shoots is especially important for establishing tissue culture derived shoots (Moss *et al.* 1988, Thiruvengadam and Jayabalan 2000). Different experiments were conducted with half and full strength of MS medium supplemented with different concentrations of auxins (NAA, IAA and IBA) singly.

In case of multiplication of shoots, similar observations were made by Sahoo and Chand (1998) in *V. negundo* when subcultured on MS medium supplemented with BA [(4.40 µ*M*/l) (1.0 mg/l)] and GA3 [(1.15 µ*M*/l) (0.4 mg/l)] up to 2 subcultures and then there was a gradual decline. Sahoo and Chand (1998) also 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.

Vadawale *et al.* (2006) studied that MS media supplemented with 4.4 u*M* BAP and 0.53 u*M* NAA induced an average of five shoots per node and was the best for auxillary bud proliferation of this plant. MS fortified with BA 1.0 mg/l was found to be the most effective for inducing multiple shoots from nodal explants. Best rooting was induced (93%) in excised shoots on half strength MS medium supplemented with an optimal combination of NAA (0.3 mg/l) (Afroz *et al*. 2008). Jawahar *et al.* (2008) observed that the frequency of callus induction in *V. negundo* increased with increasing concentration of IAA (0.3 mg/l) and BAP (0.3 mg/l) at optimal level; 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.

Conclusion

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. Thus the fruitful utilization of rapid clonal propagation, germplasm conservation and distribution of *Vitex nigundo,* important medicinal plant of Bangladesh, is possible.

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