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AN EFFICIENT CALLUS INDUCTION AND REGENERATION OF *VITEX NEGUNDO L.* – AN IMPORTANT MEDICINAL PLANT

***[1]Dharaneeswara Devi Reddy, [2]Sreeyapureddy Anitha and [3]Dowlathabad Muralidhara Rao**

[1]Plant Tissue Culture Laboratory, Department of Biotechnology, Sree Vidyanikethan Engineering College, A. Rangampet, Chittoor Dist – 517 102, A. P., India
[2,3]Department of Biotechnology, Sri Krishnadevaraya University, Anantapuramu-515 055, Andhra Pradesh, India

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ABSTRACT

An efficient protocol was described for rapid callus induction and regeneration from internodal explants of *Vitex negundo* (Verbenaceae) which is a woody, aromatic and medicinal plant. For callus induction and regeneration, Particular plant growth regulators were used, which are auxins such as 2,4-D and NAA in combination with cytokinin BAP. The explants were cultured on Murashige and Skoog (MS, 1962) medium augmented with different concentrations of auxins and cytokinins individually and in mixture of combinations for callus induction. Highest frequency and weight of brown-greenish white callus was obtained in internodal explants cultured on MS medium supplemented with 2.0 mgL^{-1} 2,4-D and 1.0 mgL^{-1} NAA in combination with 2.0 mgL^{-1} BAP. The present study also describes plant regeneration from *in vitro* derived callus. The callus was cultured on MS medium treated with BAP alone or in combination with NAA for organogenesis. Higher frequency and maximum number of shoots were obtained from callus culture on MS medium augmented with 2.0 mgL^{-1} BAP in combination with 0.75 mgL^{-1} NAA. All *in vitro* raised shoots were transferred to rooting MS medium supplemented with different concentration of IBA and NAA for root induction and the best roots were obtained on MS medium enriched with 0.5 mgL^{-1} IBA. The rooted plantlets were transferred to net pot containing autoclaved soil and vermiculite in 1:1 ratio for hardening in green house conditions.

INTRODUCTION

Vitex negundo Linn. (Family–*Verbenaceae)* is a deciduous, woody aromatic and an important medicinal plant (shrub). It grows to a height of 2-5 meters in wastelands, found in South-Asian countries and distributed throughout India (Wealth of India, 1976). All of its parts are used in Ayurvedic and Unani systems of medicine. The extracts from its leaves (Dharmasiri MG *et al.,* 2003) and seeds (Chawla AS *et al.,* 1992) are widely used for rheumatism and inflammation of joints and also reported to have wound healing, medicinal and insecticidal properties (Ramesh P *et al.*, 1986; En-shun, 2009). The plant parts are reported to have anti-bacterial (Samy *et al.,* 1998); anti-fungal (Sathiamoorthy *et al.,* 2007; Aswar *et al.,* 2009); and anti-viral properties (Nguyen-Pouplin *et al.,* 2007). It has potential to cure sprained joints, vomiting, intermittent fever, skin diseases, intestinal bilharzias, headache, amoebiasis, stomach ache and in view of its clinical potential, it acts as a good analgesic, anti-inflammatory and anti-convulsive agent (Tandon *et al.,* 2006b, Gupta GK and Tandon VR, 2005). Conventionally, *Vitex negundo* plants can be propagated by seeds or vegetative cuttings, but requires more space and large amount of plant material. Rapid asexual multiplication can be achieved by either enhancing the release of axillary buds from the apical dominance (shoot tip culture) or through somatic embryogenesis

Corresponding author: Dharaneeswara Devi Reddy
Plant Tissue Culture laboratory, Dept. of Biotechnology, Sree Vidyanikethan- Engineering College, A. Rangampet, Chittoor Dist – 517 102, A. P., India

(Murashige, 1974). Micropropagation or Rapid clonal propagation is the most advanced technique in plant tissue culture technology. In recent years, there has been an increased interest in the use of *in vitro* culture technique as a viable tool for mass multiplication and germplasm conservation of rare, endangered, aromatic and medicinal plants. Only few reports are available on the Micropropagation of *Vitex negundo*. Callus free multiple shoot formation from axillary meristem explants in MS medium with BAP (2 mgL^{-1}) and GA_3 (0.4 mgL^{-1}) has been reported by Sahoo and Chand, 1998. The mass (shoot) multiplication from nodal explants in MS medium with BAP (1.5 mgL^{-1}) and NAA (0.1 mgL^{-1}) has been achieved by Thiruvengadam and Narayansamipillai, 2000.

In the area of Plant biotechnology, plant tissue culture is a technique which supports the production of an enormous array of phytochemical compounds under aseptic conditions (Faisal, M *et al.,* 2007). Callus and cell culture carries an exceptional role for producing medicinal and bioactive compounds in large-scale from plants (Rao and Ravishankar, 2002). *In vitro* established Callus culture can be used for observation of different physiological and morphogenic responses; and used for isolation of economically valuable phytochemicals, which can avoid the collection of plant materials from the natural sources (Ogita *et al.*, 2009). Earlier number of attempts has been made *in vitro* propagation of *Vitex negundo*, still considerable efforts are being required to find an efficient protocol for callus induction and regeneration under aseptic conditions. Hence, the present study reports on the establishment of an efficient protocol for callus induction and regeneration of *Vitex negundo* using internodal segments as the explants.

MATERIALS AND METHODS

Plant Material, Explants Preparation and Sterilization

Internodal parts of plant material were collected from a healthy *Vitex negundo* plant and they were washed under running tap water for 3 minutes to remove partially bound contaminants and traces of dust. The plant material is chopped to about 3-5 cms in length containing two or three internodes. These explants were treated with a combination of blitox fungicide (2 gL^{-1}) and 2 drops of tween-20 detergent solution for 45 minutes followed by rinsing thrice with distilled water. After that the surface sterilization of explants is carried out in laminar air flow chamber as per the following procedure. The explants were washed with sterile distilled water for 1 minute. Then the explants were treated with ascorbic acid and citric acid solution for 2 minutes followed by rinsing twice with sterile distilled water. At this time, the explants were soaked in 70% alcohol for 30 sec followed by rinsing with sterile distilled water twice. Now, the explants were treated with a combination of 0.12% $HgCl_2$ surfactant and 1 drop of tween-20 solution for 8 minutes followed by rinsing with sterile distilled water thrice and subsequently the explants were trimmed to size of 3 to 4 cms.

Medium and Treatments

After sterilization, the trimmed explants were cultured on Murashige & Skoog, 1962 medium supplemented with 30 gL^{-1} sucrose and different concentration of BAP (0.25 mgL^{-1}, 0.5 mgL^{-1}, 1.0 mgL^{-1} & 2 mgL^{-1}) in combination with 2,4-D (0.25 mgL^{-1}, 0.5 mgL^{-1}, 1.0 mgL^{-1} & 2 mgL^{-1}) and NAA (0.125 mgL^{-1}, 0.25 mgL^{-1}, 0.5 mgL^{-1} & 1.00 mgL^{-1}) for callus induction. The callus culture was maintained by subculture at 3-4 weeks intervals to fresh medium with the same composition. The callus was treated on MS medium supplemented with different concentrations of 6-Benzyl Amino Purine (0.25 mgL^{-1}, 0.75 mgL^{-1}, 1.0 mgL^{-1}, 2.0 mgL^{-1}) and NAA (0.125 mgL^{-1}, 0.25 mgL^{-1}, 0.50 mgL^{-1}, 0.75 mgL^{-1}) for shoot induction. Then, the shoots were treated on MS medium containing different concentrations of NAA (0.2 mgL^{-1} and 0.40 mgL^{-1}) and IBA (0.25 and 0.50 mgL^{-1}) for induction of rooting system. All the media were adjusted to pH 5.7 using 0.1 N NaOH or 0.1 N HCl prior to adding agar and then distributed into culture vessels, plugged with polypropylene caps. The media was sterilized at 121^{o}C for 15 minutes under pressure of 15 lbs. The media is allowed to cool in the culture room until further use.

Culture Conditions

All the culture vessels were incubated at 24±2° C and 55% of relative humidity under 16/8 hours (light/dark) photoperiod with light supplied by the white fluorescent tubes at 3000 Lux in growth chamber.

Acclimatization

Healthy shoots with well developed roots were transferred to net pots containing autoclaved soil and vermiculite; and humidity is maintained through misting device inside the green house.

Experiment Design and Data Collection

Experiments were set up in completely randomized design. Eight cultures were raised for each treatment and all experiments were conducted thrice. Data was collected on the potency of the callus, number of shoots, number of roots, etc.

RESULTS AND DISCUSSION

Plant growth regulators are play a vital role in production and development of undifferentiated mass of tissue (callus) from the differentiated tissue (explants), and plant regeneration under most favorable culture conditions (Serhantova V *et al.,* 2004). Virtually, all plants require either auxin or cytokinin alone or combination of auxin and cytokinin in MS medium for callus induction and regeneration from different explants (Chan L K *et al.,* 2008). The effect of phytohormones on development of *in vitro* callus in *Vitex negundo* L was reported by Nirmalakumari A *et al.,* 2006. To induce callus, all the cultured vessels were wrapped and incubated under dark conditions in growth chamber. Callus initiation along the cut portions of internodal explants was observed after 7-18 days depending on the combination and concentrations of hormones of 2,4-D, NAA and BAP in MS medium. Results were obtained within two weeks of culture period where in 100% of callogenic response, faster calli

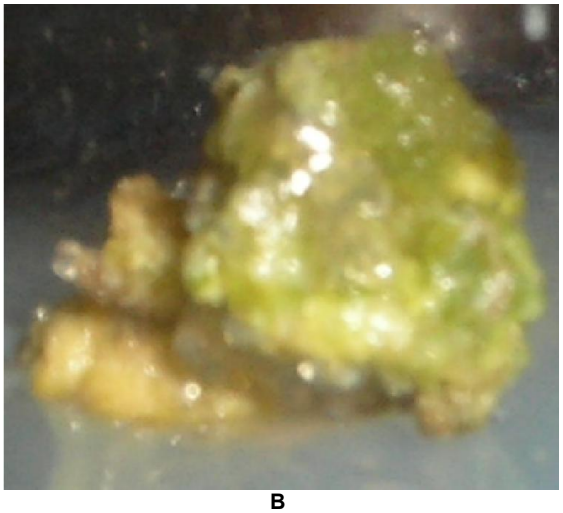
Table 1. Effect of MS medium supplemented with 2,4-D, NAA and BAP at different concentrations on callus induction from internodal explants of *Vitex negundo*

Plant Growth Regulators (mgL^{-1})			No. of explants inoculated	Day of initial callus formation	Callog-enesis	Callus weight (mg)	Callus Morphology
2,4-D	NAA	BAP					
0	0	0	8	0	-	0	No Callus was observed
0.25	-	-	8	18^{j}	-	0.001^{p}	White
0.50	-	-	8	16^{i}	-	0.097 ± 0.001^{n}	White
1.00	-	-	8	12^{f}	+	0.121 ± 0.001^{l}	White
2.00	-	-	8	9^{c}	+	0.216 ± 0.002^{j}	White
0.25	0.125	-	8	13^{g}	+	0.328 ± 0.001^{h}	White, Compact
0.50	0.25	-	8	11^{e}	++	0.480 ± 0.002^{g}	White, Compact
1.00	0.50	-	8	9^{c}	++	0.604 ± 0.003^{f}	White, Compact
2.00	1.00	-	8	8^{b}	+++	0.897 ± 0.001^{e}	Greenish, Compact
0.25	0.125	0.25	8	11^{e}	++	0.980 ± 0.002^{d}	White-Yellowish
0.50	0.25	0.50	8	10^{d}	++	1.049 ± 0.002^{c}	White-brownish
1.00	0.50	1.00	8	8^{b}	+++	1.225 ± 0.002^{b}	Brown-white, greenish
2.00	1.00	2.00	8	7^{a}	+++	1.842 ± 0.010^{a}	Brown-white, greenish
-	0.125	0.25	8	14^{h}	-	0.086 ± 0.001^{o}	Greenish-white
-	0.25	0.50	8	12^{f}	+	0.104 ± 0.001^{m}	Greenish-white
-	0.50	1.00	8	12^{f}	+	0.192 ± 0.002^{k}	Greenish-white
-	1.00	2.00	8	11^{e}	++	0.227 ± 0.002^{i}	Greenish-white

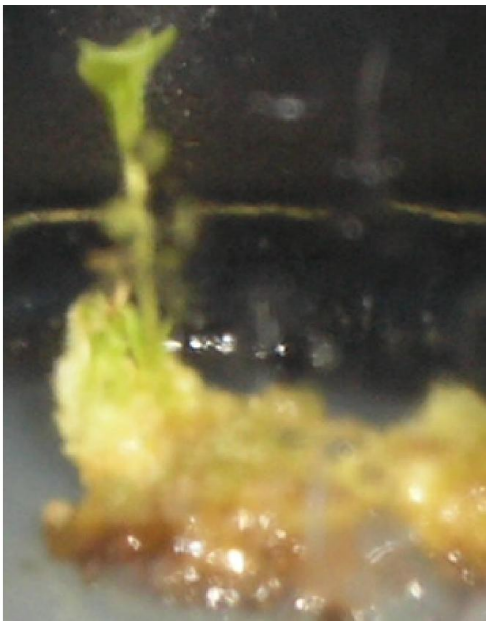
Callogenesis: -No Response; + Poor Response; ++ Good Response, +++ Better Response
Results: Mean ± Standard Error of the Mean (SEM) of 20 replicates

development, vigorous in calli growth, friable and embryonic callus with brown greenish-white colour was observed on MS medium treated with 2.0 mgL^{-1} 2, 4-D, 1.00 mgL^{-1} NAA and 2.0 mgL^{-1} BAP (Fig.-1 A & B).

A



B



C

D

E

F

Figure 1. *In vitro* establishment stages of *Vitex negundo* (A) Induction of callus from intermodal explants of *Vitex negundo* in MS medium treated with 2,4-D (2 mgL^{-1}), NAA (1 mgL^{-1}) and BAP (2 mgL^{-1}); (B) Friable, embryonic with brown-greenish white callus; (C) Proliferation of shoot from basal callus of *Vitex negundo;* (D) Proliferation of multiple shoots and root proliferation from the shoots; (E) Hardening of *Vitex negundo* plantlets in net pots under green house conditions and (F) Hardened single net pot of *Vitex negundo*.

Of the various concentrations of 2,4-D, NAA and BAP tested, the maximum callus formation was observed on MS medium supplemented with 2.0 mgL^{-1} 2,4-D, 1.0 mgL^{1} NAA and 2.0 mgL^{-1} BAP from the internodal explants with the shortest time of 7 days (Table-1). The calli formed were friable, brown-white and greenish in color (Figure 1B) with the highest callus weight of 1.842±0.010 mg (Table-1). Whereas indication of callogenesis was observed on MS medium supplemented with 0.25 mgL^{-1} 2,4-D, 0.125 mgL^{-1} NAA and 0.25 mgL^{-1} BAP with the lowest callus weight of 0.980±0.002 mg, in white-yellowish colour (Table-1). Possibly, this may be due to the plant specificity towards the suitable combinations of plant growth regulators in MS medium, while induction was slower or no callus was induced in the less appropriate medium. To enhance the callus induction, the combinations between auxin and cytokinin has been widely used and was proven to show better callogenic response compared to either auxin or cytokinine alone. Consequently, the internodal explants cultured on MS medium supplemented with 2,4-D alone showed the poor response compared to the combinations of BAP, 2,4-D and NAA at different concentrations (Table-1).

Among the different combinations of Auxin and cytokinin ratio tested, 2,4-D, NAA and BAP is proved to be superior in terms of inducing and developing high frequency of brown-greenish white callus from internodal explants of *Vitex negundo* L. The well developed brown-greenish white calli was derived from internodal explants of *Vitex negundo* and the callus was used for organogenesis. The calli was subcultured in MS medium fortified with different concentration and combinations of plant growth regulators such as BAP and NAA for shoot initiation. The cultured vessels were incubated in growth chamber for organogenesis of callus *i.e.* from undifferentiated mass of tissue to differentiated tissue under favorable conditions. Shoot proliferation was observed from the callus surface after thirteen days of subculture Figure 1c. Among these, the calli sub-cultured on MS medium supplemented with 2 mgL^{-1} BAP and 0.75 mgL^{-1} NAA initiated highest percentage (95%) of shooting response (Table-2 and Figure 1D).

Table 2. Effect of MS medium supplemented with NAA and BAP at different concentrations on shoot initiation from *in vitro* derived callus of *Vitex negundo*

Plant Growth Regulators(mgL^{-1})		% of Shoot response	Number of shoots per callus
NAA	BAP		
0.125	-	38%	0.8 ± 0.0^{l}
0.25	-	44%	1.4 ± 0.1^{k}
0.50	-	54%	1.6 ± 0.1^{j}
0.75	-	62%	1.9 ± 0.1^{i}
-	0.25	41%	1.0 ± 0.1^{h}
-	0.75	59%	1.9 ± 1.2^{g}
-	1.0	64%	2.2 ± 0.1^{f}
-	2.0	71%	2.6 ± 0.2^{e}
0.125	0.25	68%	3.1 ± 0.3^{d}
0.25	0.75	74%	3.8 ± 0.2^{c}
0.50	1.0	83%	4.5 ± 0.2^{b}
0.75	2.0	95%	7.0 ± 0.3^{a}

Results: Mean ± Standard Error of the Mean (SEM) of 20 replicas

Table 3. Effect of MS medium supplemented with NAA and IBA at different concentrations on root initiation from *in vitro* regenerated shoots of *Vitex negundo*

Plant Growth Regulators(mgL^{-1})		% of Root response	Number of roots per plantlet
NAA	IBA		
0.2	-	51%	0.8 ± 0.1^{f}
0.4	-	59%	1.2 ± 0.1^{e}
-	0.25	69%	4.1 ± 0.3^{c}
-	0.50	87%	6.4 ± 0.2^{a}
0.2	0.25	62%	4.2 ± 0.2^{b}
0.4	0.50	74%	3.7 ± 0.3^{d}

Results: Mean ± Standard Error of the Mean (SEM) of 20 replicas

The low frequency of shoot initiation was observed in MS medium supplemented with 0.25 mgL^{-1} BAP at lower concentration, further the frequency of shoot initiation enhances with increase in BAP concentrations (0.25 mgL^{-1}–2.0 $mgL^{-1)}$. In the present study, the shoot bud differentiation and maximum percentage of shoot induction was observed on MS medium supplemented with cytokinin (BAP) in combination with auxin (NAA) (Figure 1D). The well developed shoots were excised and transferred to MS medium augmented with different concentrations of plant growth regulators such as NAA and IBA. Among these, the plantlets inoculated on MS medium supplemented with IBA 0.5 mgL^{-1} yielded highest percentage of rooting (87%) compare to the NAA supplemented MS medium (Table-3). IBA has been reportedly useful for inducing rooting in a variety of plants (Ramanayake *et al.,* 2008). Ramanayake *et al.,* (2006) reported that, a rooting medium with IBA 3 mgL^{-1} was optimum for rooting induction and MS medium. Faisal *et al.,* (2007) reported that IBA is superior to IAA and NAA in induction of roots for *Tylophora indica.* In the present study, the MS medium treated with IBA gave the maximum percentage of root induction. The well rooted plantlets were graded from the culture vessels, washed and transferred to net pots containing autoclaved soil and vermiculite in 1:1 ratio and hardened to green house conditions Figure 1E and 1F.

Conclusion

An efficient protocol has been developed for inducing *in vitro* callus and regeneration of *Vitex negundo*. In the present study, enhanced *in vitro* callus and regeneration of plants with combination of plant growth regulators such as NAA, 2,4-D, IBA and BAP was observed. This will be helpful in understanding the callogenesis and organogenesis through the internodes and to facilitate the mass propagation of *Vitex negundo*.

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