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# Impacts of cytokinins and auxins on organogenesis and somatic embryogenesis of *Vitex negundo* var. *Negundo* L. and evaluation of genetic fidelity

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## ABSTRACT

The present study was aimed to produce an efficient direct and indirect micro propagation system for *Vitex negundo* var. *negundo* Linn. a medicinally important plant using nodal, leaves and intermodal segments as explants. Adventitious proliferation was obtained from *Vitex negundo* var. *negundo* nodal segments inoculated on Murashige and Skoog's basal medium with 3% sucrose and augmented with 6-Benzyl Amino purine. Highest frequency of shoot proliferation (75.3 ± 0.03) was observed in Murashige and Skoog's medium augmented with 6.66µM of 6-Benzyl Amino purine and the maximum number per explants (8.2 ± 0.79) was also obtained in very same concentration. Maximum percentage of callus formation (stem 75.2 ± 0.90; leaves 72.3 ± 0.38) was obtained on Murashige and Skoog's basal medium supplemented with 3% and 2,4-Dichlorophenoxy acetic acid 2.26µM. Maximum percentage (80.2 ± 0.2) of shoot proliferation from the inter-nodal derived calli was achieved on Murashige and Skoog's medium augmented with 3% sucrose and 6-Benzyl Amino purine 4.44µM in combination with α-Naphthalene Acetic Acid 2.69µM. Three month old leaves calli cultures were produced the pro-embryogenic calli on the surface of the callus tissue. The maximum percentage (69.8 ± 0.96) of pro-embryogenic calli obtained in Murashige and Skoog's medium supplemented with 2,4-Dichlorophenoxy acetic acid (4.52µM) combination with Kinetin (2.32µM). Murashige and Skoog's medium supplemented with Kinetin (4.65µM) combination with 2,4-Dichlorophenoxy acetic acid (2.26µM) showed the maximum proliferation of embryo like structure. Maximum percentage of shootlet proliferation (60.8 ± 0.58) obtained on Murashige and Skoog's medium augmented with 6-Benzyl Amino purine 6.66µM alone. Half strength Murashige and Skoog's medium with 3% sucrose augmented with Indole 3-Butyric Acid 4.92µM showed the maximum frequency (85.3 ± 0.03) of root formation from the *in vitro* derived shootlets. The micro propagated plantlets genetic uniformity was confirmed through the isozyme analysis. The *in vitro* raised plants were hardened then transferred to field for re-establishment. 

## KEYWORDS

*In vitro*;
Calli;
Micropropgation;
Isozyme;
Iso-peroxidase.

## INTRODUCTION

*Vitex negundo* var. *negundo* Linn. belonging to a gamopetalous family '*Verbenaceae*' is a medicinally important plant distributed throughout India. The plant is bitter, acrid, thermogenic, expectorant, carminative, digestive, stomachic, anodyne, anti-inflammatory, antiseptic, cephalic, alterant, antipyretic, diuretic, emmenagogue, depurative, rejuvenating, ophthalmic, vulnerary and tonic. The roots used in vitiated conditions of *vata, kapha, jajvara*, cephalalgia, sprains, orchitis, gout, splenohepatomegaly, otorrhoea, inflammations ulcers, cephalagia, otalgia, arthritis, inflammation, dyspepsia, colic, verminosis, flatulence, dysentery, uropathy, wounds, bronchitis, cough, malarial fever, haemorrhoids, dysmenorrhoea, leprosy, dermatopathy, ophthalmopathy and general debility. The bark is used in vitiated conditions of *vata*, odontalgia, verminosis and ophthalmopathy. The flowers used in diarrhoea, cholera, fever, haemorrhages, hepatopathy and cardic disorders[1]. The whole plant is having great demand on the market due to its medicinal value. So, a rapid production system of medicinal plants is required to provide continuous supply of plants for scientific research into phytochemistry and medicinal efficacy. In view of the increasing demand for *Vitex negundo* var. *negundo*, there is a need to develop new approaches for efficient propagation. The conventional methods were applied for multiplication of this medicinal plant the results are not satisfactory, *in vitro* propagation is an alternative tool for large scale multiplication and may increase the number of propagules for cultivation as well as aid the replacement of natural populations plant tissue culture techniques offer a powerful tool for mass multiplication of medicinally important plant species[2-5,7]. Preliminary work on the tissue culture of *Vitex negundo* has been reported by Thiruvengadam and Jayabalan[27] and Jawahar[28]. This study describes the results of the study taken up for developing an effective, reproducible and simple protocol for clonal multiplication and conservation of *Vitex negundo* var. *negundo*. In addition the present study emphasized on organogenesis and somatic embryogenesis induction, the auxin and cytokinin influence on the morphogenetic development and evaluation of genetic uniformity and variation using iso-peroxides also included.

## MATERIALS AND METHODS

Plants of *Vitex negundo* var. *negundo* L. were collected from Kollimalai, Salem, Tamil Nadu, India was established in the green house and herbal garden. Young shoots were harvested and washed with running tap water and surface sterilized in 0.1 (w/v) $HgCl_2$ solutions for one minute. After rinsing 3-4 times with sterile distilled water, leaves, stem nodes, internodes were cut into smaller segments (1cm) used as the explants. The explants were placed horizontally as well as vertically on solid basal Murashige and Skoog[6] (MS) medium supplemented with 3% sucrose, 0.6% (w/v) agar (Hi-Media, Mumbai) and different concentration and combination of IBA, BAP, KIN, 2,4-D, IAA, NAA and IBA for direct and indirect organogenesis. The pH of the medium was adjusted to 5.8 before autoclaving at 12°C for 15min. The cultures were incubated at 25 ± 2°C under cool fluorescent light (2000lux 14 hr photoperiod). The inter-nodal and leaves derived callus was sub-cultured onto MS medium supplemented with (BAP, Kin, NAA, 2,4-D, etc.) different concentration and combinations of PGRs for morphogenesis. The proembryogeneic calli were sub-cultured onto MS medium augmented with different concentrations and combinations of PGRs for embryo formation and shootlets proliferation. For rooting, the *in vitro* raised plantlets were transferred to the ½MS medium augmented with different concentrations and combinations of auxins. For hardening, the *in vitro* raised plantlets were removed from culture, washed thoroughly with tap water planted in small polycups filled with sterile garden soil (3:1), covered by unperforatedpolybags, and hardened for 4 weeks in a mist chamber before transfer to field.

For Genetic fidelity analysis, the leaves were collected, washed and grinded in apre-chilledpestle and mortar into a fine paste by adding 500 µl of 1M Phosphate buffer (pH 7.0) the slurry was centrifuged for 10 min in a refrigerated centrifuge at 10,000rpm at 4°C. The supernatant (extract) stored in eppendorf tubes in freezer and subsequently used for isoenzyme (peroxidase) analysis[2-4,7]. For peroxidase isoenzyme the PAGE electrophoresis was performed by Anbalagan method (1999). After running the gel was stained with 0.1% 0-Dianisidine, acetate buffer (pH 4.7) and 30% hydrogen peroxide and incubated in

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dark for half an hour. Then the gel was fixed in 7% acetic acid for 15 min[7]. The enzyme peroxidases appeared as dark orange bands. Based on the banding profile the peroxidase analysis and RF values were calculated.

## RESULTS AND DISCUSSIONS

The medium (MS) augmented with different concentrations of BAP was used for multiple shoots emergence from the nodal segments. After 7 days of inoculation, the buds were started for shoot proliferation the effect of BAP on shoot multiplication from nodal explants is shown in TABLE 1. The medium containing BAP (6.66μM) induced maximum number of multiple shoots (8.2 ± 0.79) with maximum percentage (75.3 ± 0.03). BAP induced multiple shoot formation was reported by other workers in *Baliospermum axillare*[8,23], *Solanum Surattense*[9] and *Baliospermum montanum*[2-4,7]. Our present study results directly coincided with the previous worker reports. When the concentration of BAP increased there is reduction in number of multiple shoots. The minimum number (3.8 ± 1.03) of shootlets observed in MS medium augmented

**TABLE 1 : Influence of plant growth regulators on organogenesis and somatic embryogenesis of *Vitex negundo* L**

| MS medium with plant growth regulators (μM) | | | | | | % of shoot lets formation±S.E. | Mean length of shootslets ±S.E. | Mean no.of shootlets/Explants± S.E. | % of Callus induction±S.E. | | % Pro embryo calli formation±S.E | % Somatic embryogenesis formation | % of Rootlets formation/shootlet |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BAP | KIN | 2,4-D | IBA | IAA | NAA | | | | Leaves | Inter-Nodal | | | |
| Direct regeneration from nodal segments | | | | | | | | | | | | | |
| 2.22 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 65.3±0.20 | 3.4±0.42 | 4.0±0.78* | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 4.44 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 71.4±0.10 | 4.5±0.50 | 7.0±0.87* | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 6.66 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 75.3±0.03 | 6.0±0.68 | 8.2±0.79* | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 8.88 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 70.8±0.20 | 7.1±0.73 | 6.6±0.56* | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 11.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 68.1±0.01 | 5.0±0.64 | 5.0±0.02* | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Callus induction | | | | | | | | | | | | | |
| 0.0 | 0.0 | 2.26 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 72.3±0.3 | 75.2±0.9 | 0.0 | 0.0 | 0.0 |
| 0.0 | 0.0 | 4.52 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 65.8±0.2 | 70.8±0.5 | 0.0 | 0.0 | 0.0 |
| 0.0 | 0.0 | 6.78 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 60.3±0.3 | 65.6±0.5 | 0.0 | 0.0 | 0.0 |
| 0.0 | 2.32 | 9.04 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 55.4±0.4 | 60.4±0.6 | 0.0 | 0.0 | 0.0 |
| 0.0 | 2.32 | 12.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 53.7±0.1 | 57.8±0.4 | 0.0 | 0.0 | 0.0 |
| Indirect regeneration via calli | | | | | | | | | | | | | |
| 4.44 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 75.3±0.1 | 2.8±0.1 | 4.6±0.5** | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 4.44 | 0.0 | 0.0 | 0.0 | 0.0 | 2.69 | 80.2±0.2 | 3.0±0.4 | 5.5±0.2** | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 6.66 | 0.0 | 0.0 | 0.0 | 0.0 | 2.69 | 67.3±0.1 | 3.1±0.1 | 3.2±0.3** | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 4.44 | 0.0 | 0.0 | 0.0 | 2.85 | 0.0 | 72.5±0.3 | 3.2±0.1 | 4.5±0.4** | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 0.0 | 4.65 | 0.0 | 0.0 | 2.85 | 0.0 | - | - | - | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 0.0 | 4.65 | 0.0 | 0.0 | 0.0 | 2.69 | - | - | - | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 4.44 | 0.0 | 2.26 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 23.8±0.36 | 43.6±0.65 | 0.0 |
| 0.0 | 2.32 | 4.52 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 69.8±0.96 | 58.6±0.93 | 0.0 |
| 6.66 | 0.0 | 6.78 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 32.6±0.81 | 45.7±0.45 | 0.0 |
| 0.0 | 4.65 | 2.26 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 56.3±0.74 | 72.3±0.68 | 0.0 |
| 4.44 | 0.0 | 4.52 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 46.7±0.85 | 56.3±0.72 | 0.0 |
| *In vitro* Rootlet formation *** | | | | | | | | | | | | | |
| 0.0 | 0.0 | 0.0 | 4.92 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 83 .3±0.1 |
| 0.0 | 0.0 | 0.0 | 0.0 | 5.71 | 2.69 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 70.8±0.02 |
| 0.0 | 0.0 | 0.0 | 9.84 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 78.3±0.01 |

***Sign indicates the number of shootlets per nodal segments; **Sign indicates the number of shootlets per calli; ***Sign indicates the ½ strength MS medium**

with Kin (4.65μM) in combination with NAA (2.69μM) (TABLE 1).

Callus was initiated from the leaves and stem explants on the MS basal medium supplemented with different concentrations and combinations of 2,4-D and Kin (TABLE 1). The maximum percentage of friable callus (75.2 ± 0.9 in stem and 72.3 ± 0.3 in leaf) proliferation was obtained on MS medium supplemented with 2, 4-D (2.26μM). The callus obtained from leaf was white in color on MS medium supplemented with 2,4-D (2.26μM) where as the callus obtained from the inter-nodal segment was light pale yellow. The effect of 2,4-D in the induction of callus was also reported by Manickam *et al*.[2-4,10,23] in *Withania somnifera* and the effect of Kin was reported by Rout *et al*.[11] in *plumbago zeylanica.* In the present study we obtained maximum percentage of callus on MS medium supplemented with 2,4-D. Our result was directly consonance with the Manickam *et al*.[2-4,10,23] observation on *Withania somnifera*. Calli obtained from the inter-nodal and leaf explants were tested for shoot regeneration (TABLE 1). The maximum number of shootlets regeneration (5.5 ± 0.2) were observed from MS medium supplemented with 444μM BAP in combination with 2.69μM NAA. The maximum number of shoot and lengths were decreased in the lower concentration of Kin (4.65μM), IAA (2.85μM) and NAA (2.69μM). The maximum number of shoots and the maximum percentage (80.2 ± 0.2) of shoot regeneration were also observed in MS medium BAP and NAA. Similar report was reported by Manickam *et al*.[2-4,10,23] in *Withania somnifera*; pawar *et al*.[9], and Sudha and Seeni[12] in *Adhatoda beddomi*.

The *in vitro* inoculated leaves produced a white friable callus on the wounded surface. The maximum (72.3 ± 0.38) induction of calli obtained in Murashige and Skoog's medium supplemented with 2,4-D 2.26μM (TABLE 1). The in vitro induced calli were sub cultured periodically for the stock maintenance and other analysis. The callus structure and color varies depending on the growth regulators used. Explants cultured (Three month old calli) on media containing 2,4-D with addition of kinetin gave rise to nodular embryogenic callus, which was yellow and loosely packed on the surface of the creamy layer of the calli, microscopical examinations confirmed the pre-embrogeneic development, where as the nodular embryogenic callus developed on media enriched in low concentration of 2,4-D in combination with BAP was cream in color and compactly packed, Tiwari et al.[13] also observed the embryogenic development in 2,4-D combination with BAP supplemented media. Famelaer et al.[14] and Ptak and Bach[15,20] obtained similar results in *in vitro* tulip culture and Ma *et al*.[16] observed the somatic embryo development on 2,4-D alone supplemented media. MS media fortified with 4.0mg $L^{-1}$ 2,4-D in combination with 0.5mg $L^{-1}$ BAP was found to be the most effective in onion[13]. In the present study, maximum percentage (69.8 ± 0.96) of pro-embryogenic calli obtained in Murashige and Skoog's medium supplemented with 2,4-D (4.52μM) combination with Kin (2.32μM) (TABLE 1). In the present study BAP was replaced by kinetin, the observation revealed that the cytokinin alone or in combination with auxin promotes the embryogeic development. The present study results coincided with the previous observations of Sagare et al.[17] Kaparakis and Alderson[18]. The Murashige and Skoog's medium supplemented with Kin (4.65μM) combination with 2,4-D (2.26μM) showed the maximum proliferation (72.3 ± 0.68) of embryo like structure (TABLE 1). In Onion, the reduced level of 2.0mg $L^{-1}$ 2,4-D in combination with 0.5mg $L^{-1}$ BAP promoted faster development of embryos in the subsequent subculture[13]. Similar to that in the present study also, the reduced level of 2,4-D and increased Kinetin level promoted the development and multiplication of the embryo. Somatic embryos are generally promoted by auxins either alone[16] or in combination with cytokinins[13,17,18]. In the present study also the somatic embryo induced by the stimulation of 2,4-D and Kinetin. Embryogenic callus formation was accompanied by the development of colorless callus, which lacked capability for somatic embryogenesis. The multiplication of embryogenic callus and the formation of somatic embryogenesis occurred on the initial media. In the present study, the globular embryos of *Vitex negundo* var. *negundo*, irrespective of their origin, were able to convert into well developed plants on media containing BAP alone. Ptak and Bach[15,20] observed the globular embryos of tulip, were able to convert into well-developed plants on media containing 5μM BA and 0.5μM NAA. In the present study the BAP alone promoted the development. Media con-

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taining NAA were also used by Hulscher et al.[19] for tulip shoot regeneration via organogenesis and by Bach[15,20] for germination of somatic embryos of the hyacinth. The maximum percentage of shootlets proliferation (60.8 ± 0.58) obtained on Murashige and Skoog's medium augmented with 6-benzyl aminopurine 6.66µM alone (data's not included). BAP is considered one of the best cytokinin for achieving the multiplication and micropropagation plants[5,21,22]. The *in vitro* raised shootlets were transferred to half strength MS medium with different concentrations and combinations of IBA, IAA and NAA for rooting (TABLE 1). The maximum number (7.5± 0.23)of rootless were observed on MS medium augmented with IBA (4.92µM). The present study report was directly coincide with previous workers observations Johnson and Manickam[2-4,7] in *Baliospermum montanum*; Sivasubramanian et al.[23] in*plectranthus vetiveroides* and Johnson[2-4,7] in *Adenia hondala* and *Justicia jendurussa* they got maximum number of rootlets on the very same medium and hormone (½MS medium augmented with IBA 4.92µM) and their results supported the present observation.

After 30 days of rooting, *in vitro* raised plantlets were hardened in polycups containing a mixture of sterile garden soil: sand (3:1), covered with polypropylene bags and irrigated with 10x diluted MS liquid medium. The plants were kept in a culture room for 15 days. 90% of plants were successfully established in polycups. After 15 days the polycups hardened plants, were transferred to pots and kept in green house. 90% of plants were well established in the green house condition. After one month, the plants were transferred to the field. About 80% of plants were established in the field.

The genetic conformity test was carried out between the mother plant, *in vitro* raised by nodal segments, callus mediated plant and somatic embryo mediated plantlet it was noticed that there is some change in the banding pattern in callus mediated and somatic embryo mediated regeneration. Callus mediated plant showed the maximum number of bands compared to the mother plant, nodal and shoots tip derived plants. The nodal derived plantlets and mother plants showed similar banding profiles and no variation between the mother and daughter plants; but calli mediated and somatic embryo raised plantlets showed the additional banding

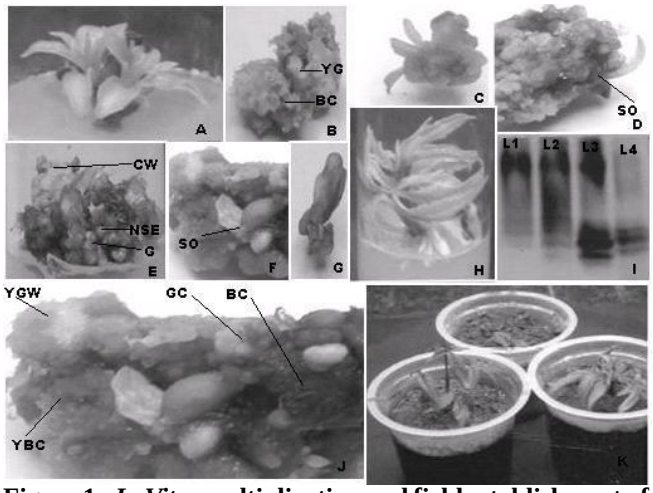


**Figure 1 : *In Vitro* multiplication and field establishment of *Vitex negundo* L. Multiple shootlets initial stage-nodal segments explants. Inter-nodal segment derived calli (YG-yellowish green calli; BC-brown calli) Shoot regeneration on *in vitro* derived stem calli. Different types of calli from leaves and shootlet origination from leaves derived calli (SO-shoot originated from leaves derived calli base). E. Different kind of embryogenic calli (CW-creamy white calli; NSE-nodular pre-embryogenic calli with somatic embryo; G-globular embryo). F. shoot originated from the globular embryo. G. somatic embryo regenerated shootlet. H. *In vitro* derivedplantlet with shootlet and rootlets. I. iso-peroxidase analysis on mother plant (L1), stem calli mediatedplantlet (L2), somatic embryo mediated plantlet (L3) and leaves derived calli mediated plantlet (L4). J. different type of calli derived from *in vitro* cultured leaves and globular type embryo formation different stages (YGW-yellowish green white creamy callus; YBC-yellowish brown calli; GC-green calli and BC-brown calli. K. different developmental stages of hardened plants inpoly cups**

profiles compared to the mother plants. It confirmed the somoclonal variations presence in the calli mediated and somatic embryo derived plantlets (Figure 1). Similar kind of analysis was performed by Mondal et al.[24], Merce et al.[25] and Johnson[2-4,7] with different plants, they also confirmed the somoclonal variations using isozyme and protein analysis. The use of isozymes as genetic markers as increased dramatically over the last decades as it has number of important advantages over more conventional morphological markers[26]. The present study isozyme results also strengthen the application of isozymes as a marker in systematic and plant breeding programme.

## CONCLUSION

In the present study, we successfully developed the protocol for *in vitro* propagation of the medicinally im-

portant plant *Vitex negundo* var. *negundo* (L) using nodal, leaves and inter-nodal segments as explants. This will provides a substantial base and efficient protocol for future biotechnological, phytochemical and bio-efficacy research. In addition, the isozyme banding pattern provided the taxonomical markers for the mother plant, *in vitro* derived plantlets, this will applied in the plant systematic studies as a biochemical marker.

## ABBREVIATIONS

MS-Murashige and Skoog's medium; pGRs-plant growth regulators; BAP-Benzyl aminopurine; IAA-Indole-3-acetic acid; IBA-Indole-3-butyric acid; NAA-α-naphthalene acetic acid; 2,4-D-2,4-dichlorophenoxy acetic acid; 2,4,5-T-2,4,5-trichlorophenoxy acetic acid.

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