Plant Archives Vol. 23, No. 2, 2023 pp. 73-79 e-ISSN:2581-6063 (online), ISSN:0972-5210

Plant Archives

Journal homepage: http://www.plantarchives.org
DOI Url : https://doi.org/10.51470/PLANTARCHIVES.2023.v23.no2.013

IN VITRO ORGANOGENESIS AND HIGH-FREQUENCY PLANT REGENERATION IN *VITEX NEGUNDO* (L) USING THIDIAZURON

Garg G.*, Singh S. and Chaudhary S.
School of Biotechnology, Gautam Buddha University, Greater Noida, Uttar Pradesh, India
*Corresponding author e-mail: gunjangarg@gbu.ac.in
(Date of Receiving : 11-05-2023; Date of Acceptance : 13-06-2023)

ABSTRACT

In this work, a simple, efficient and repeatable protocol was developed for in vitro regeneration via- callus- mediated organogenesis of *Vitex negundo* using nodal explants. Effects of basal medium and plant growth regulators, with nodal explants were studied. We report optimum response of callus induction from nodal segment explants in the MS medium containing 1.0 mg/l TDZ and 1.5 mg/l BAP. When BAP and TDZ individually or in combination with 2, 4-D and NAA were employed for callus regeneration, shoot and embryo like structure (ELSs) were noted in the callus from nodal explants. The best responses of callus per explants were given by TDZ with NAA. ELSs/explants were ascertained on MS medium with 1.0 mg/l TDZ. TDZ 0.1mg/l and BAP 1.5 mg/l along with NAA were found to perform well for shoot regeneration via callus from nodal explants. Our research data will be significant for in vitro mass propagation of *V. negundo* via somatic embryogenesis. It can consider as vital and promising off shoot protocol for the conservation of medicinally significance and endangered plant. This work is the first report of a consistent, definitive, and unique protocol for *V. negundo* regeneration, paving the way for the in vitro preservation of such significant genetic resources and also further allied systems based on such callus-based or embryo-based approaches.
Keywords: *Vitex negundo*, nodes, embryo-like structures (ELSs), organogenesis

Introduction

The genus *Vitex* (Verbenaceae) encompasses approximately 250 species around the sphere (Shanwaz and Shyam, 2023). Bioactive compounds present in the different species of vitex plant, traditionally used to cure many diseases like rheumatoid arthritis, sinuses, toothache, headache and ulcers. These compounds have potential to cure skin infection, digestive troubles, skin-ulcers, bronchitis, diabetes and gonorrhea. They also showed anticancer, antipyretic, anti-inflammatory, insecticidal properties. The leaves are used as mosquito repellent. Extract of leaves showed anti-bacterial, anti-pyretic, anti-hyperglycemic, anti-histaminic, anti-implantation properties. It also used in the treatment of catarrhal fever. In the current time Vitex species attain spike attention due to their pharmacological properties. *Vitex negundo* (L.) is an woody, aromatic-perennial shrub, commonly called as Nisinda or Chinese-chaste tree or Five-leaved chaste tree. It is indigenous to tropical eastern and southern part of Africa and Asia, and widely used in folk medicine. Different parts of *V. negundo* (L.) were used in treatment of bronchitis, asthma and gastric troubles (Sahayaraj K, Ravi 2008). This plant is the rich source of active compounds such as betulinic acid and ursolic acid (Thombre *et al*., 2013). Chemically leaves contain alkaloids, flavonoides such as flavones, luteoline-7-glycoside, casticin, iridoid glycosides, essential oil and additional components like vitamin C, carotene, glycol-nonital, benzoic acid, β-sitosterol and C-glycoside (Praveen *et al*., 2010). *V. negundo* (L.) is propagated by seed and root suckers. Conventional propagation through vegetative cutting faces issues in the form of slow multiplication. Due to the medicinal property of *V. negundo*, exploration of research has been increased in the last few years in the pharmaceutical and phytochemical stream, resulted over harvesting of this perennial-plant from its natural habitat. It resulted *V. negundo* to become in the list of endangered plants. This enforces to develop an alternative-efficient methods of propagation through which large scale production of this plant would be possible. There are many methods of micro-propagation in plant tissue culture for the different species of *Vitex*, but most of them showed poor regeneration and survival tendency. There is very limit literature on the somatic embryogenesis process in in-vitro condition in Vitex species. On the basis of the literature reviews and continuous increasing pharmaceutical industrial demand of *V. negundo*, this plant was selected for tissue culture research. The aim of present research work was to develop simple and rapid methods of plant regeneration through *in vitro* organogenesis and callus mediated somatic embryogenesis for *Vitex negundo* an important, aromatic-perennial shrub.

Materials and Methods

Collection, identification and sterilization of explant

Vitex negundo (L.) is small-perennial-shrub. Sample of *Vitex negundo* collected from the Central Institute of Medicinal and aromatic plant (CIMAP), Lucknow and maintained in herbal-garden, Gautam Buddha University (Campus), Greater Noida. Identification of plant sample was done by National herbarium of cultivated plants (NHCP), Division of plant exploration and germplasm collection,

ICAR-NBPGR, PUSA Campus, New Delhi-110012. Young shoot segments with one node were used as explants for the establishment of primary cultures (Fig.1). For the sterilization of explants, collected young nodal shoots washed properly by running tap water for 20 min, followed by presoaked in 1% Tween 20 (liquid detergent) for 20-25 min, and sinking in 70% (v/v) ethanol for 5 min. Explants were then surface sterilized with 0.1% (w/v) mercuric chloride for 5-7 min. These explants were finally rinsed 3-4 times with sterile double distilled water inside a laminar hood. Each time leave the explant in sterilized double distilled water for 5-7 minutes to remove the sterilizing agent. The surface sterilized explants were incised into appropriate size (1.0-1.5 cm in length) containing a single node with an axillary bud and inoculated vertically on the MS medium (Murashige and Skoog, 1962) containing different plant growth regulators (PGRs).

Culture media, plant growth regulators (PGRs) and culture conditions

In the present study, MS medium supplemented with sucrose (3% *w/v*) and pH 5.7-5.8 before the addition of agar-agar (0.8% *w/v*) and autoclaving at 121 °C at 15lbs pressure for 15-20 min. The PGRs used in the experiments were auxin including 2, 4-dichlorophenoxyacetic acid (2, 4-D), naphthalene acetic acid (NAA); cytokinin 6-benzyladenine (BAP), thidiazuron (TDZ) alone and in combination of auxins (Table.1). Before the inoculation, media containing culture plates were exposed to UV light for 25-30 minutes under laminar hood. Inoculation of explants was done in laminar air cabinet in aseptic conditions in the culture plates of diameter 7.0 x 8.0 cm (diameter), containing 10-15 ml medium. All the culture plates were incubated in cultured room at 22 ± 2°C under 16-18 hrs photoperiod at 3000 lux intensity of light (40W white fluorescent tubes), with the 55-60% relative humidity (RH). Regenerated new shoot induced in the culture media in *in vitro* conditions were further used as explants for shoot regeneration. Cultures were observed at every third day and recorded the data on axillary bud-shoot proliferation and callus induction.

Axillary Shoot Proliferation and Somatic Embryo-like Analogous Structure (ELSs) from Nodal Segments

For inducing the axillary bud-shoot proliferation, callus induction and ELSs formation in *in-vitro* conditions, inoculate the single node of young shoot segments (1.0-1.5 cm in length) with axillary bud vertically onto MS medium containing various PGRs (Table 1) along with the control as MS medium with no PGRs. For further multiplication of the cultures, samples with high differentiation ability were subculture on the same respective media. Following 6-7 weeks of culture, shoot regeneration, callus formation and ELSs development were assessed and compared.

Data record and analysis

The cultures are maintained as *in-vitro* organogenesis cultures from February to April 2023 in the culture room of plant biotechnology laboratory, school of biotechnology, Gautam Buddha University, Greater Noida. *In vitro* initiation of shoot and callus formation, the percentage of survival of explant and organogenesis response was recorded. For the confirmation of results, the entire experiment repeated thrice and per treatment we used five replicates.

Table 1: Effect of PGRs on induced morphogenesis from nodal explant of *V. negundo*

S. No.	Media code	Growth regulator concentration with MS medium	Numbers of nodes per culture plates	Shoot initiation	% Response of shoot initiation	Embryoid like structure (ELSs)	% response of ELSs	Degree of callus initiation	% response of callus initiation	Response time (in days)
1.	**C0**	MS0	10	-	-	-	-	-	-	-
2.	**C1**	MS + 2,4-D (1.0 mg/l)	10	+	20%	-	-	-	-	16
3.	**C2**	MS + 2,4-D (1.5 mg/l)	10	+	20%	-	-	-	-	15
4.	**C3**	MS + 2,4-D (2.0 mg/l)	10	+	30%	-	-	-	-	15
5.	**C4**	MS + NAA (1.0 mg/l)	10	+	20%	-	-	-	-	14
6.	**C5**	MS + NAA (1.5 mg/l)	10	+	40%	-	-	-	-	16
7.	**C6**	MS + NAA (2.0 mg/l)	10	+	30%	-	-	-	-	13
8.	**C7**	MS + BAP (1.0 mg/l)	10	+++	80%	-	-	-	-	13
9.	**C8**	MS + BAP (1.5 mg/l)	10	+++	100%	-	-	-	-	10
10.	**C9**	MS + BAP (2.0 mg/l)	10	++	60%	-	-	-	-	14
11.	**C10**	MS + TDZ (1.0 mg/l)	10	-	-	+++	90%	-	-	10
12.	**C11**	MS + TDZ (1.5 mg/l)	10	-	-	+	30%	-	-	15
13.	**C12**	MS + TDZ (2.0 mg/l)	10	-	-	+	20%	-	-	14
14.	**C13**	MS+ 2,4-D (2mg/l) +BAP (0.25mg/l)	10	-	-	-	-	+	50%	27
15.	**C14**	MS+ 2,4-D (2mg/l) +BAP (0.5mg/l)	10	-	-	-	-	+	40%	28
16.	**C15**	MS+ 2,4-D (2mg/l) +TDZ (0.25mg/l)	10	-	-	-	-	++	70%	24
17.	**C16**	MS+ 2,4-D (2mg/l) +TDZ (0.5mg/l)	10	-	-	-	-	++	70%	22
18.	**C17**	MS+ NAA (2mg/l) +BAP (0.25mg/l)	10	-	-	-	-	+	50%	25
19.	**C18**	MS+ NAA (2mg/l) +BAP (0.5mg/l)	10	-	-	-	-	++	70%	27
20.	**C19**	MS+ NAA (2mg/l) +TDZ (0.25mg/l)	10	-	-	-	-	++	60%	30
21.	**C20**	MS+ NAA (2mg/l) +TDZ (0.5mg/l)	10	-	-	-	-	+++	90%	23

Response: + slight (10-50%), ++ moderate (60-70%); +++ profuse (80-100%); - no response (0%)

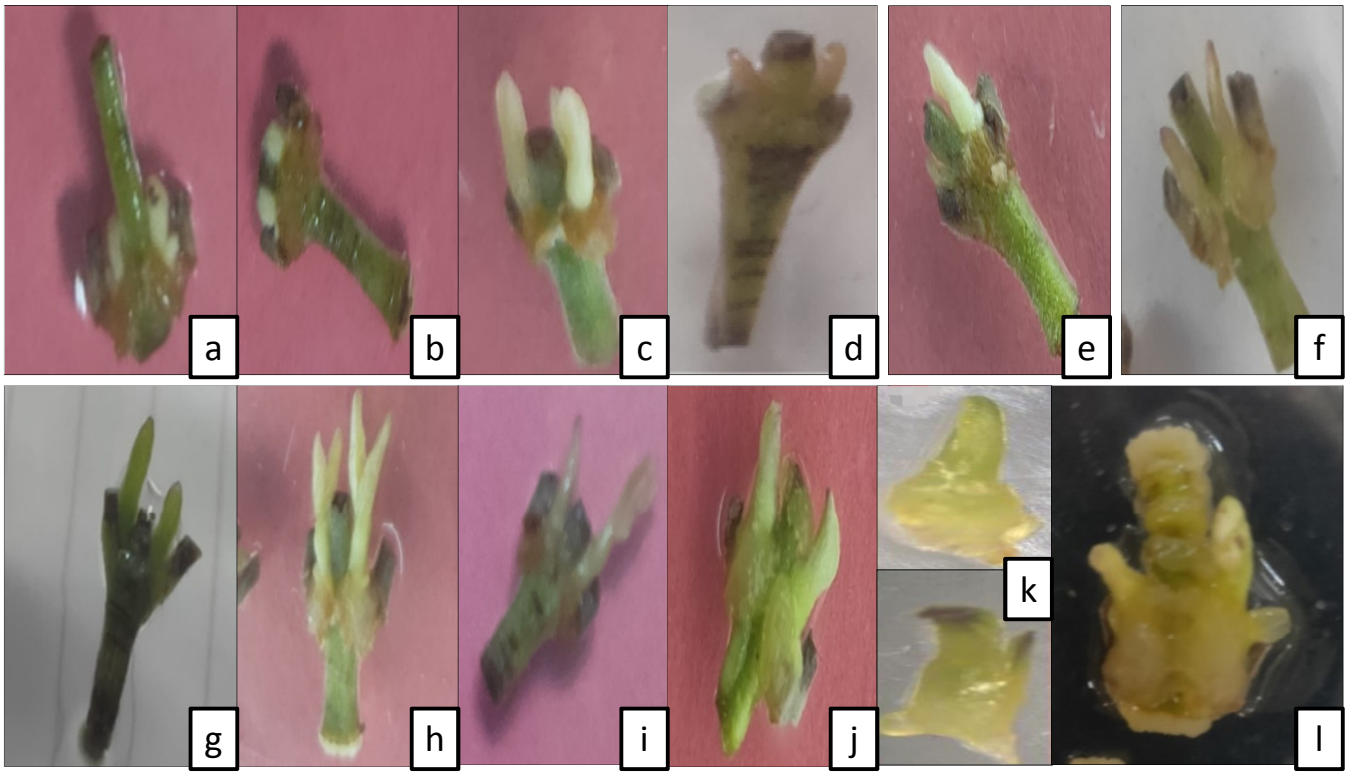

Fig. 1: Direct organogenesis from the nodal explants of *Vitex negundo:* (a-f) C1-C6 media resulted whitish shoot initiation; (g-i) C7-C9 media showed light greenish axillary buds proliferation; (j-l) C10 media showed development of light-greenish globular and torpedo shaped ELSs

Table 2: Effect of different PGRs on *in vitro* callus induction from nodal explants in *V. negundo* after 35 days of culture

Media code	Growth regulator concentration with MS medium	Degree of callus initiation	% Response of Callus initiation	Response time (in days)	Morphology of callus	Callus color
C13	MS+ 2,4-D (2mg/l) +BAP (0.25mg/l)	+	50%	27	Compact	Whitish-yellow
C14	MS+ 2,4-D (2mg/l) +BAP (0.5mg/l)	+	40%	28	Wet/ loose	brownish
C15	MS+ 2,4-D (2mg/l) +TDZ (0.25mg/l)	++	70%	24	Compact	Light green
C16	MS+ 2,4-D (2mg/l) +TDZ (0.5mg/l)	++	70%	22	Granular	off white-Creamy
C17	MS+ NAA (2mg/l) +BAP (0.25mg/l)	+	50%	25	Compact	Greenish white
C18	MS+ NAA (2mg/l) +BAP (0.5mg/l)	++	70%	27	Friable-granular	Greenish white
C19	MS+ NAA (2mg/l) +TDZ (0.25mg/l)	++	60%	30	Granular-compact	Greenish white
C20	MS+ NAA (2mg/l) +TDZ (0.5mg/l)	+++	90%	23	Friable-granular	Greenish white

2, 4-Di-chlorophenoxyacetic acid (2, 4-D), Naphthalene acetic acid (NAA); 6-Benzyladenine(BAP), Thidiazuron (TDZ)

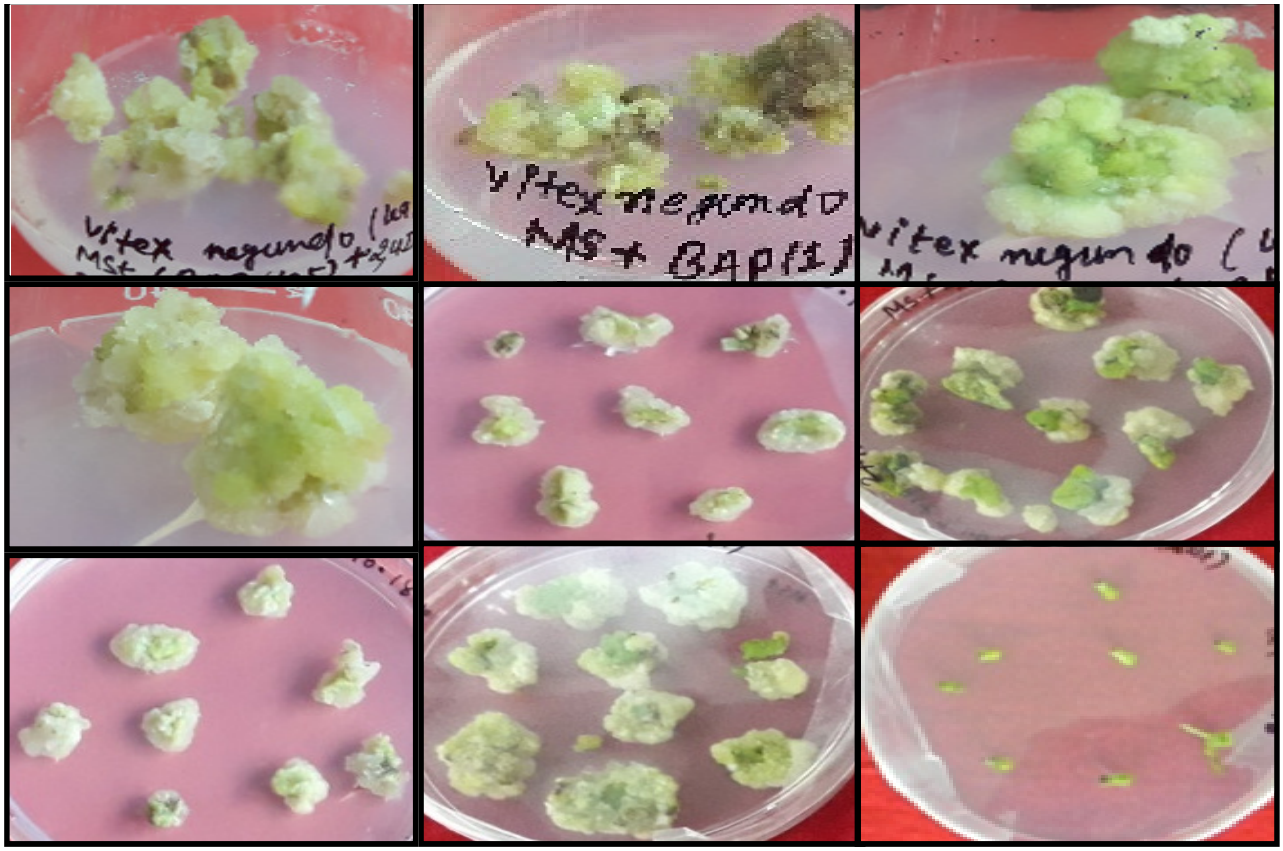

Fig. 2: Interaction effect of different PGRs on *in vitro* callus induction from nodal explants in *V. negundo* after 35 days of culture: (a-d) browning of callus in culture media (C13-C16) supplemented with cytokinine (BAP and TDZ) in combination with 2, 4 D; (e-h) Compact greenish-white/ yellow callus observed in culture media (C17-C20) supplemented with cytokinine (BAP and TDZ) in combination with NAA; (i) no callus formation on explants cultured in C0 culture media

Table 3: Effect of cytokinins for regeneration of shoot from callus of *V. negundo* after 4-5 weeks of culture

Media code	Growth regulator concentration with MS medium	No. of callus/ treatment	Shooting response from callus	% of shooting response from callus	Response time (in days)
C7	MS + BAP (1.0 mg/l)	4	3	75%	20
C8	MS + BAP (1.5 mg/l)	4	3	75%	18
C9	MS + BAP (2.0 mg/l)	4	3	75%	15
C10	MS + TDZ (1.0 mg/l)	4	2	50%	18
C11	MS + TDZ (1.5 mg/l)	4	3	75%	15
C12	MS + TDZ (2.0 mg/l)	4	4	100%	10

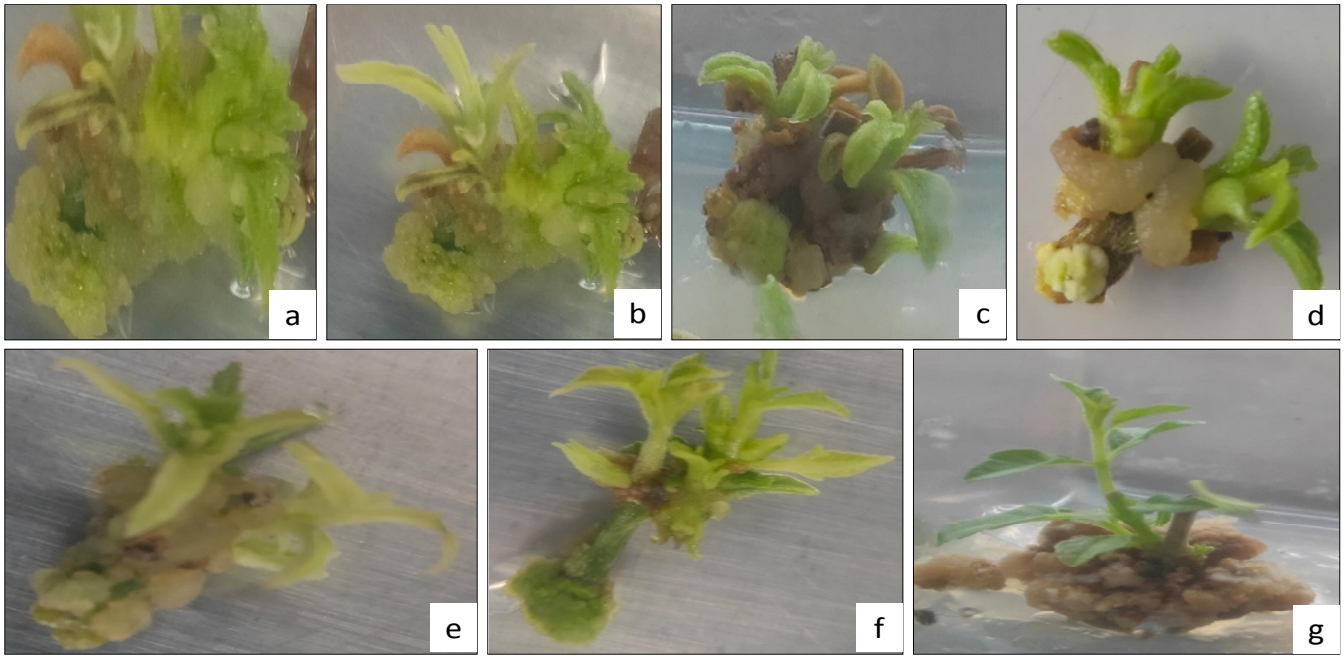

Fig. 3 Indirect organogenesis and plant regeneration in *Vitex negundo* (a-b) Induction of callus from the cut end of nodes in MS medium supplemented with NAA (2mg/l) +TDZ (0.5mg/l); (c-f) Multiple shoots formation the callus after the 10 days from inoculation (media supplemented with 2 mg/l TDZ); (g) Shoot elongation and development of leaves after 4 weak of culture

Results

Morphogenetic responses of nodal explants under the influence of PGRs

The morphogenetic responses of nodal explants evaluated to the various concentrations (1.0, 1.5 and 2.0 mg/l) of cytokinins (BAP and TDZ) and auxins (2, 4 D and NAA) showed a marked effect on multiple shoot induction. Nodal explants cultured on MS basal medium served as control, did not promote axillary bud initiation whereas, one nodal segment proliferated into axillary shoots in the presence of growth regulators favored axillary bud induction and considerable enhancement in response was achieved with the application of different concentrations of BAP. Of the different concentrations of cytokinins tested, BAP (1.5 mg/l) was found to be given best result compared with the TDZ in respect to the shoot initiation and axillary buds proliferation. Robust shoots with dark-green leaves were induced within 10 days of culture with 100% maximum frequency in the medium supplemented with 1.5 mg/l BAP (Table 1). A light-green callus was induced on the cut surface of the nodes in 10-12 days in the culture medium supplemented with 1.0 mg/l TDZ only. Later, the formation of a protuberance, globular and torpedo shaped structures (Fig.1j-l) were documented in the consecutive time interval. Expanded the culturing time of nodal segment-callus on the same culture media we observed the somatic embryo-like analogous structure (ELSs) from its surface. A higher level of TDZ (1.5-2.0 mg/l) did not significantly increase the frequency of induction of ELSs (Table 3).

In our research experiment we inoculated nodal explants on MS medium supplemented with different concentrations of cytokinins (TDZ and BAP) individually or in combinations with auxins (2, 4 D and NAA) and we observed that additions of auxins with selected cytokinine enhanced callus formation along with the regeneration of adventitious shoot from callus in *V. negundo*. The combination of TDZ (0.5 mg/l) along with NAA (2.0 mg/l) augmented the callus formation (90%/ nodal-explant) in the medium (Table 2). After 4-5 weeks, the greenish and friable callus almost covered the explants with some adventitious shoots (Figs. 2-3). In our experiment we observed that one-third of the nodal explants callus turned brown (Table 2) at wounded site surfaces with the enlargement of callus. The browning rate was conspicuously more in the culture media supplemented with selected concentrations of cytokinine (BAP and TDZ) in combination with 2, 4 D.

Discussion

In vitro propagation via the multiplication of axillary shoots is an efficient system for mass producing true-to-type plants (Faisal and Alatar, 2022). This approach sees the widespread usage of nodal segment explants as reported for *Plectranthus zeylanicus* (Fonseka *et al*., 2019) *Plectranthus esculentus* (Kujeke *et al*., 2020), Brahmi (Faisal *et al*., 2018), and *Rumex pictus* (El-Shafey *et al*., 2019). Results along the similar line have been reported for the genus Campomanesia. Wróbel *et al*. (2022), Machado *et al*. (2020) and El-Shafey *et al*. (2019) showed the importance of adding PGRs for axillary shoot proliferation from Cannabis, Campomanesia and *Rumex pictus* nodal explants. In our experiment we reported that MS medium that lacked PGRs displayed no shoots. Out of the two selected types of cytokinins (BAP and TDZ), only one concentration of cytokinins i.e. BAP 1.5 mg/l effectively sustained shoot

regeneration. The number of shoots regeneration capacity was decline with the increasing cytokinine concentration above their optimum level. BAP 1.5 mg/l was the most effective concentration among the other concentrations of cytokinins employed in axillary shoot regeneration in *Vitex negundo* to that reported by Nowakowska *et al*. (2020).

TDZ is believed to be the best synthetic cytokinin for the formation of somatic embryos and regeneration of numerous plant systems (Nowakowska, 2022, Kirtis and Aasim, 2019). In our research we observed that high concentration of TDZ (2.0 mg/l) induces the regeneration capacity of shoots from the callus, whereas lower concentration of TDZ (1.0 mg/l) promoted formation of ELSs, supported the views of Ghosh *et al*. (2018) and Xu *et al*. (2022). Hyper-hydricity exhibited regeneration of shoots from the callus in the culture media supplemented with TDZ at 2.0 mg/l. Kadota and Niimi (2003) reported that hyper-hydricity of the cell or tissue is influenced by different types and concentrations of cytokinine. We observed the same findings in our results. The optimum concentration and type of PGRs is different for different explants (Ahmad *et al*., 2018). In our results, most optimal cytokinine concentration for nodal segment is 1.5 mg/l of BAP and it showed robust-axillary shoots proliferation (100%). Furthermore, 0.5 mg/l of TDZ, in combination with 2 mg/l of NAA was suitable for callusing (90%) from the node as explant. TDZ 1.0 mg/l were the best media for induction of ELSs.

In most of the methods of micro-propagation in different Vitex species, nodes or leaves as an explants, gave better and successful results as compared to the other explants like petiole, roots, internodes, shoot tip etc. (Muthu and Narayanasamypillai (2000). The experimental data and literature is quite scarce on somatic embryogenesis in *Vitex* species except for *Vitex doniana*. Colombe *et al*. (2015) was the first to report somatic embryogenesis in *Vitex doniana* leaf explants. In our study, we observed embryo-like structures (ELSs) from the nodal explants on MS medium containing cytokinins (1.0 mg/l TDZ), which later formed, globular and torpedo shaped structures, that finally developed into real shoots (Table 1, Fig. 1j-l). TDZ and NAA were found as the best with regard to callus induction as opposed to BAP plus NAA. For the successful establishment of the *in vitro* cultures, elimination of explant browning is an essential process. Long exposure of explants to the high concentrations of PGRs or any other physical conditions, resulted necrosis and browning of the explant, which subsequently leads to death of cells or explants. However in our investigation, we observed no browning of callus when the explant inoculated with TDZ. Opposite to the results of TDZ, we observed callus-browning in the explants inoculated with BAP, which may be assorted response of cytokinine with different explants or different plant species. In the recent years there has been developed an interest in *in vitro* biotechnological and genetic engineering approaches, which offered viable protocols for the mass multiplication and germplasm conservation of medicinally plants which are now categorized and listed into endangered and threatened plants, due to their over-exploitation for their pharmaceutical values. Our research data will be significant for in vitro mass propagation of *Vitex negundo* sp. via somatic embryogenesis. It can consider as vital and promising off-shoot protocol for the conservation of medicinally significant and endangered plants.

Conclusions

Vitex negundo is an aromatic large shrub. Different plant parts of *V. negundo* possess diverse pharmacological activities. The plant is reported to contain potent and novel therapeutic agents for scavenging of NO (nitrous oxides). Due to enormous medicinal properties, the plant has been used extensively. Therefore *in vitro* callus from leaves, stem, and inter-node etc. part have been raised using various growth regulators like auxins and in combination with cytokinins. Even though there are very limited protocols for somatic embryogenesis is available in the different species of vitex. In the present study we established *in vitro* regeneration protocols using direct method i.e. shoot induction from axillary bud and indirect method via the formation of embryo-like structures (ELSs), which will be useful for the conservation and improvement of the medicinal plants germplasm. We also suggest the further research to explore the effect of this protocol at agrobacterium mediated genetic transformation level. In conclusion more investigation are necessary to enhance the viability and germination percentage of somatic embryos and subsequently develop the procedure for the establishment of such plants in the green house. Additionally theses embryos like structures are the prospective material for the production and preservation of the artificial seeds of this vital species.

References

Ahmad, N., Faisal, M, Hussain, S.A. and Anis, M. (2018). Regulation of morphogenesis and improvement in shoot multiplication in Vitex Species using thidiazuron. Book Chapter: Thidiazuron: From Urea Derivative to Plant Growth Regulator, pp 343-349, Publisher: Springer Singapore, Hardcover ISBN978-981-10-8003-6, DOI: 10.1007/978-981-10-8004-3_18.

Banerji, J., Das, B., Chakrabarty, R. and Jha, H.C. (2020). Isolation of 4 4' dimethoxy trans stilbene and flavonoids from leaves and twigs of *Vitex negundo* L. *Indian Journal of Chemistry*, 27(6): 597-599.

Colombe, D., Jane, K., Catherine, M., Lucien, D., Christophe, K., Peter, N. and Modeste, K. (2015). Induction and regeneration of somatic embryos from *Vitex doniana* (Lamiaceae) leaf explants. *International Journal of Biotechnology and Molecular Biology Research*, 6(4): 28-34.

Du, Y.M., Cheng, F.Y. and Zhong, Y. (2020a). Induction of direct somatic embryogenesis and shoot organogenesis and histological study in tree peony (*Paeonia sect. Moutan*). *Plant Cell Tissue Organ Culture*, 141(557–570).

El-Shafey, N., Sayed, M, Ahmed, E., Hammouda, O. and Khodary, S.E. (2019). Effect of growth regulators on micro-propagation, callus induction and callus flavonoid content of *Rumex pictus* Forssk. *Egypt Journal of Botany*, 59: 293–302.

Faisal, M. and Alatar, A. (2022). Establishment of an efficient *in vitro* propagation method for a sustainable supply of *Plectranthus amboinicus* and genetic homogeneity using flow cytometry and SPAR Markers. *Horticulture*, 8(693).

Faisal, M., Alatar, A., El-Sheikh, M., Abdel-Salam, E.M. and Qahtan, A.A. (2018). Thidiazuron induced *in vitro* morphogenesis for sustainable supply of genetically true quality plantlets of Brahmi. *Indian Crops Production*, 118: 173–179.

Fonseka, D., Wickramaarachchi, W. and Situge. C. (2019). Mass production of *Plectranthus zeylanicus*: Avaluable medicinal and aromatic plant with a future value. *International Journal of Minor Fruits, Medicinal and Aromatic Plants*, 5(15–20).

Ghosh, A., Lgamberdiev, A. and Debnath, S. (2018). Thidiazuron-induced somatic embryogenesis and changes of antioxidant properties in tissue cultures of half-high blueberry plants. *Science Report*, 8:16978,

Kadota, M. and Niimi, Y. (2003). Effects of cytokinin types and their concentrations on shoot proliferation and hyper hydricity in *in-vitro* pear cultivar shoots. *Plant Cell, Tissue and Organ Culture*, 72: 261–265.

Kirtis, A. and Aasim, M. (2019). Thidiazuron (TDZ) Induced in vitro axillary shoot regeneration of desi chikpea (*Cicer arietinum* L.). *Journal of Applied Biological Sciences*, 13(1): 17-20, E-ISSN: 2146-0108.

Kujeke, G.T., Chitendera, T.C, Masekesa, R.T., Mazarura, U., Ngadze, E., Rugare, J.T., Matikiti, A. (2020). Micropropagation of living stone Potato (*Plectranthus esculentus*), *Advances in Agriculture*, 8364153.

Machado, J.S., Degenhardt, J., Maia, F.R. and Quoirin, M. (2020). Micropropagation of *Campomanesia xanthocarpa* (Myrtaceae), a medicinal tree from the Brazilian Atlantic Forest. *Trees*, 34: 791–799.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. Plant Physiology, 15: 473-497.

Muthu, T. and Narayanasamypillai, J. (2000). Mass Propagation of *Vitex negundo* L. *in-vitro*. *Journal of Plant Biotechnology*, 2(3): 151-155.

Nowakowska, M., Pavlovic, Z., Nowicki, M., Sarah, L. and Robert, N.T. (2020). *In vitro* propagation of an endangered *Helianthus verticillatus* by axillary bud proliferation, *Plants*, 9(6): 712.

Nowakowska, K., Anna Pinkowska, A., Siedlecka, E. and Pacholczak, A. (2022). The effect of cytokinins on shoot proliferation, biochemical changes and genetic stability of Rhododendron '*Kazimierz odnowiciel'* in the *in vitro* cultures. *Plant Cell, Tissue and Organ Culture*, 149: 675–684.

Shanwaz, M.M. and Shyam, P. (2023). Synthesis of silver nano particles from *Vitex negundo* plant by green method and their bactericidal effects, 12(2): 59.

Sharma, P., Vidyasagar, G., Singh, S., Ghule, S. and Kumar, B. (2010). Anti-diarrhoeal activity of leaf extract of *Celosia argentea* in experimentally induced diarrhea in rats. *Journal of Advanced Pharma Technology Research*, 1(1): 41–48.

Usmanu, H., Danfodiyo, V., Ngweh, A., Collins, A. and Oven-seri (2023). Green synthesis of silver nanoparticles from *Parinaricura tellifolia* methanol stem bark extract and evaluation of antioxidant and antimicrobial activities. *Tropical Journal of Natural Product Research*, DOI:10.26538/tjnpr/v7i3.

Wrobel, T., Dreger, M., Wielgus, K. and Słomski, R. (2022). Modified nodal cuttings and shoot tips protocol forrapid regeneration of *Cannabis sativa* L. *Journal of Natural Fibers*, 19: 536–545.

Xu, L., Cheng, F. and Zhong, Y. (2022). Efficient plant regeneration via meristematic nodule culture in *Paeonia ostii*. *Plant Cell, Tissue and Organ Culture*, 149: 599–608.