

Research Article

# In vitro morphogenesis and conservation of the medicinal plant *Vitex negundo* L. through nodal explants

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## Abstract

*Vitex negundo* L. is an important medicinal plant widely used in traditional medicine and is valued for its diverse pharmacological properties. The present study was undertaken to develop an efficient in vitro regeneration and multiplication protocol for *V. negundo* using nodal explants and to establish a suitable approach for ex situ conservation. Nodal explants were cultured on Murashige and Skoog (MS) medium supplemented with different concentrations of cytokinin's: 6-benzylaminopurine (BAP), kinetin (KIN), and thidiazuron (TDZ), either individually or in combination with auxin indole-3-acetic acid (IAA). Among the cytokinin's tested, TDZ was the most effective for shoot induction. MS medium containing 2.0 mg L<sup>-1</sup> TDZ produced 92.6% shoot induction, with an average of 10.2 shoots per explant. Further optimization using auxin – cytokinin combinations demonstrated that MS medium supplemented with 2.0 mg L<sup>-1</sup> TDZ and 0.5 mg L<sup>-1</sup> IAA was highly effective, producing up to 96.3% shoot induction and, 26.6 shoots per explant. The regenerated shoots were subjected to root on half-strength MS media containing IBA. The highest rooting response was obtained on half-strength MS medium containing 0.75 mg L<sup>-1</sup> IBA, with 100% root induction, 12.8 roots per shoot, and a mean root length of 4.9 cm. The rooted plantlets were successfully hardened and acclimatized, with 100% survival reported under field conditions. The developed protocol provides an efficient method for rapid multiplication and ex situ conservation of *V. negundo* and may facilitate the production of quality planting material for medicinal plant cultivation.



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## 1. INTRODUCTION

Medicinal plants constitute an important source of therapeutic compounds and have traditionally contributed substantially to human healthcare. India possesses considerable medicinal plant diversity because of its varied geographical, climatic, and ecological conditions. However, excessive collection, destructive harvesting, habitat loss, fragmentation, and other anthropogenic pressures have contributed to the depletion of valuable medicinal plant resources [1]. The present investigation emphasizes the importance of integrating in situ and ex situ conservation approaches, with plant tissue culture providing an important tool for ex situ conservation and rapid multiplication. Micropropagation offers several advantages over conventional propagation, including rapid multiplication of selected genotypes, production of relatively uniform planting material, and propagation independent of seasonal limitations and conservation of valuable germplasm [2]. These advantages are particularly relevant to medicinal plants because overexploitation may threaten their natural populations.

*Vitex negundo* L. is an important medicinal tree that occurs widely in tropical areas around the globe. Different parts of the plant have traditionally been associated with several medicinal applications, viz., anti-inflammatory, antibacterial, antifungal, hepatoprotective, and insecticidal activities, and possess important bioactive constituents, including betulinic acid, ursolic acid and  $\beta$ -sitosterol [3,4].

Although *V. negundo* can be propagated through seeds, stem cuttings are not recommended since poor, seed germination and slow or limited survival of vegetative cuttings [5]. Therefore, conventional propagation methods are considered inadequate for meeting the demand for planting material. Several earlier studies have demonstrated the potential of nodal explants for micropropagation of *V. negundo* with BAP, KIN, and TDZ, and combinations of cytokinins with auxins have been investigated for shoot induction and multiplication. Sahoo and Chand [6], Ahmed and Anis [7], Kumar et al. [8], Xue et al. [9], Garg et al. [10] reported moderate to good in vitro regeneration studies with successful rooting and acclimatization of regenerated plants, demonstrating the suitability of tissue culture for propagation and conservation of the species.



Therefore, the objective of the present study was to optimize suitable and potential plant growth regulator combinations for the in vitro response of nodal explants of *V. negundo* and to develop an efficient and rapid regeneration system through direct organogenesis.

## 2. MATERIALS AND METHODS

### 2.1. Plant material:

The juvenile nodal explants of *V. negundo* L. (an aromatic deciduous shrub/semi-evergreen small tree) were collected from the Horticulture Division of Andhra University, Visakhapatnam, Andhra Pradesh, India.

### 2.2. Culture medium:

Murashige and Skoog [11] medium was used as the basal culture medium. The medium contained MS macro and micronutrients, vitamins, and amino acids. The pH was adjusted to 5.6–5.8 before autoclaving at 1.04 kg/cm<sup>2</sup> pressure and 121°C temperature for 15–20 minutes. The medium contained 3% sucrose and 0.8% agar as carbon sources and gelling agents, respectively.

### 2.3. Effect of plant growth regulators for shoot induction:

The basal MS media was supplemented with different concentrations and combinations of cytokinins viz., BAP (0.5mgL<sup>-1</sup> to 3mgL<sup>-1</sup>), KIN (0.5mgL<sup>-1</sup> to 3mgL<sup>-1</sup>) and TDZ (0.5mgL<sup>-1</sup> to 3mgL<sup>-1</sup>) were evaluated. Optimized cytokinin concentrations were subsequently combined with auxin IAA (0.5mgL<sup>-1</sup>) to determine their effect on shoot regeneration.

### 2.4. Surface sterilization and culture establishment:

The juvenile nodal explants were subjected to surface sterilization using 0.02% HgCl<sub>2</sub> mercuric chloride for 3 minutes. Then the sterilized nodal explants were inoculated aseptically onto the respective culture media. The cultures were maintained at temperature 22±2°C under a 16 h light/8 h dark photoperiod, with regular subculturing at approximately two-week intervals.

### 2.5. Shoot induction and multiplication:

Shoot induction was assessed after 4 weeks of culture by recording the percentage of explants producing shoots and the mean number of shoots produced per nodal explant by adopting the method of Iqbal et al. [12].

### 2.6. Root induction:

The well-developed shoots were excised and transferred to half-strength MS medium supplemented with different concentrations of IBA. Recorded the root induction percentage, number of roots per shoot, and root length by adopting the method of Iqbal et al. [12].

### 2.7. Acclimatization:

Rooted plantlets were carefully removed from the culture vessels and gently washed under running water to remove the adhering culture medium. The plantlets were subsequently transferred to small pots containing a sterile 1:1 mixture of sand and garden soil. The pots were covered with sterile polyethylene bags to maintain high humidity and kept in a mist chamber for two weeks. During this acclimatization period, the plantlets were irrigated periodically with inorganic salt solution. Subsequently, the hardened plantlets were transferred to earthen pots containing a 1:1:1 mixture of garden soil, sand, and farmyard manure and maintained under open field conditions.

### 2.8. Statistical analysis:

The experiments were completely randomized on factorial design; all treatments were being replicated three times with 10 replicates per treatment. The results were analyzed by one way ANOVA, and the means were separated by Duncan's multiple range test (DMRT) using SPSS (Version 10) software package.

## 3. RESULTS

### 3.1. Effect of cytokinins on shoot regeneration:

Nodal explants failed to produce shoots when cultured on MS medium without plant growth regulators, indicating the requirement for exogenous growth regulators for shoot induction. Among the three cytokinins tested, TDZ was the most effective. MS medium containing 2.0 mg L<sup>-1</sup> TDZ produced 92.6% shoot induction, with 10.2 shoots per explant (Table 1). Increasing TDZ concentration beyond the optimum resulted in reduced shoot induction and shoot multiplication. BAP was less effective than TDZ; the highest BAP response was obtained at 1.5 mg L<sup>-1</sup>, producing approximately 81% shoot induction and 6.2 shoots per explant. KIN was comparatively less effective, with 1.5 mg L<sup>-1</sup> KIN producing 68.5% shoot induction and, 4.4 shoots per explant.

### 3.2. Effect of auxin–cytokinin combinations on shoot multiplication:

The addition of auxin indole-3-acetic acid (IAA) to the optimized cytokinin concentration media improved shoot regeneration compared with cytokinin alone media. Among the combinations evaluated, TDZ and IAA produced the strongest response (Figure 1). MS medium supplemented with 2.0 mg L<sup>-1</sup> TDZ and 0.5 mg L<sup>-1</sup> IAA resulted in 96.3% shoot induction and, 26.6 shoots per explant. Increasing the TDZ concentration with IAA (0.5 mg L<sup>-1</sup>) reduced the shoot-regeneration response. The BAP + IAA combination also promoted shoot regeneration. BAP at 1.5 mg L<sup>-1</sup> combined with IAA at 0.5 mg L<sup>-1</sup> produced 84% shoot induction and, 18.5 shoots per explant. The KIN + IAA treatment produced a lower response, with a maximum shoot induction of 79.1%. The highest shoot number in this treatment was 7.2 shoots per explant.

### 3.3. Root induction:

Regenerated shoots of about 3 cm in height were transferred to half-strength MS media containing varying concentrations of IBA. Half-strength MS medium supplemented with 0.75 mg L<sup>-1</sup> IBA produced the best rooting response. The optimum treatment resulted in 100% root induction, with 12.8 roots per shoot and a mean root length of 4.9 cm (Table 2).

### 3.4. Acclimatization and field establishment:

Rooted plantlets were successfully transferred from in vitro conditions to ex vitro conditions. The rooted plants were transplanted into open field conditions after hardening showed 100% establishment. Any detectable variations in growth and morphological characteristics were not recorded in micro propagated plants.

Table (1): Invitro shoot induction from *V. negundo* nodal explants after 4 weeks of culture.

| S. no.               | Plant Growth Regulators  |                          |                          |                          | Invitro response  |                         |
|----------------------|--------------------------|--------------------------|--------------------------|--------------------------|-------------------|-------------------------|
|                      | BAP (mgL <sup>-1</sup> ) | KIN (mgL <sup>-1</sup> ) | TDZ (mgL <sup>-1</sup> ) | IAA (mgL <sup>-1</sup> ) | Shooting (%)      | Shoot no./explant       |
| 1.                   | -                        | -                        | -                        | -                        | 0.0 <sup>k</sup>  | 0.0 ± 0.0 <sup>m</sup>  |
| 2.                   | 0.5                      | -                        | -                        | -                        | 65.0 <sup>f</sup> | 3.8 ± 0.5 <sup>j</sup>  |
| 3.                   | 1.0                      | -                        | -                        | -                        | 70.8 <sup>c</sup> | 5.4 ± 0.4 <sup>i</sup>  |
| 4.                   | 1.5                      | -                        | -                        | -                        | 81.0 <sup>c</sup> | 6.2 ± 0.1 <sup>h</sup>  |
| 5.                   | 2.0                      | -                        | -                        | -                        | 75.0 <sup>d</sup> | 5.1 ± 0.6 <sup>j</sup>  |
| 6.                   | 2.5                      | -                        | -                        | -                        | 65.0 <sup>f</sup> | 4.0 ± 0.4 <sup>j</sup>  |
| 7.                   | 3.0                      | -                        | -                        | -                        | 50.0 <sup>h</sup> | 2.9 ± 0.2 <sup>k</sup>  |
| 8.                   | -                        | 0.5                      | -                        | -                        | 37.9 <sup>i</sup> | 1.5 ± 0.3 <sup>l</sup>  |
| 9.                   | -                        | 1.0                      | -                        | -                        | 48.6 <sup>h</sup> | 2.0 ± 0.2 <sup>l</sup>  |
| 10.                  | -                        | 1.5                      | -                        | -                        | 68.5 <sup>c</sup> | 4.4 ± 0.5 <sup>j</sup>  |
| 11.                  | -                        | 2.0                      | -                        | -                        | 58.0 <sup>g</sup> | 3.2 ± 0.4 <sup>k</sup>  |
| 12.                  | -                        | 2.5                      | -                        | -                        | 50.2 <sup>h</sup> | 2.8 ± 0.7 <sup>k</sup>  |
| 13.                  | -                        | 3.0                      | -                        | -                        | 46.7 <sup>i</sup> | 1.2 ± 0.9 <sup>l</sup>  |
| 14.                  | -                        | -                        | 0.5                      | -                        | 78.5 <sup>c</sup> | 5.0 ± 0.3 <sup>i</sup>  |
| 15.                  | -                        | -                        | 1.0                      | -                        | 85.0 <sup>b</sup> | 6.4 ± 0.5 <sup>h</sup>  |
| 16.                  | -                        | -                        | 1.5                      | -                        | 90.4 <sup>a</sup> | 7.3 ± 0.4 <sup>g</sup>  |
| 17.                  | -                        | -                        | 2.0                      | -                        | 92.6 <sup>a</sup> | 10.2 ± 0.1 <sup>f</sup> |
| 18.                  | -                        | -                        | 2.5                      | -                        | 90.0 <sup>a</sup> | 8.1 ± 0.2 <sup>g</sup>  |
| 19.                  | -                        | -                        | 3.0                      | -                        | 79.6 <sup>c</sup> | 4.6 ± 0.6 <sup>j</sup>  |
| 20.                  | 0.5                      | -                        | -                        | 0.5                      | 60.6 <sup>g</sup> | 10.0 ± 0.8 <sup>f</sup> |
| 21.                  | 1.0                      | -                        | -                        | 0.5                      | 68.9 <sup>c</sup> | 14.2 ± 0.1 <sup>c</sup> |
| 22.                  | 1.5                      | -                        | -                        | 0.5                      | 84.0 <sup>b</sup> | 18.5 ± 0.4 <sup>c</sup> |
| 23.                  | 2.0                      | -                        | -                        | 0.5                      | 78.4 <sup>c</sup> | 13.2 ± 0.7 <sup>c</sup> |
| 24.                  | 2.5                      | -                        | -                        | 0.5                      | 60.6 <sup>g</sup> | 9.2 ± 0.5 <sup>f</sup>  |
| 25.                  | 3.0                      | -                        | -                        | 0.5                      | 55.1 <sup>g</sup> | 6.3 ± 0.2 <sup>h</sup>  |
| 26.                  | -                        | 0.5                      | -                        | 0.5                      | 59.2 <sup>g</sup> | 3.2 ± 0.9 <sup>k</sup>  |
| 27.                  | -                        | 1.0                      | -                        | 0.5                      | 65.8 <sup>f</sup> | 5.0 ± 0.6 <sup>j</sup>  |
| 28.                  | -                        | 1.5                      | -                        | 0.5                      | 79.1 <sup>c</sup> | 7.2 ± 0.3 <sup>g</sup>  |
| 29.                  | -                        | 2.0                      | -                        | 0.5                      | 70.4 <sup>c</sup> | 6.1 ± 0.1 <sup>h</sup>  |
| 30.                  | -                        | 2.5                      | -                        | 0.5                      | 65.0 <sup>f</sup> | 4.8 ± 0.5 <sup>j</sup>  |
| 31.                  | -                        | 3.0                      | -                        | 0.5                      | 49.5 <sup>h</sup> | 2.9 ± 0.8 <sup>k</sup>  |
| 32.                  | -                        | -                        | 0.5                      | 0.5                      | 58.0 <sup>g</sup> | 9.2 ± 0.5 <sup>f</sup>  |
| 33.                  | -                        | -                        | 1.0                      | 0.5                      | 67.6 <sup>c</sup> | 15.0 ± 0.7 <sup>d</sup> |
| 34.                  | -                        | -                        | 1.5                      | 0.5                      | 80.0 <sup>c</sup> | 18.8 ± 0.2 <sup>c</sup> |
| 35.                  | -                        | -                        | 2.0                      | 0.5                      | 96.3 <sup>a</sup> | 26.6 ± 0.4 <sup>a</sup> |
| 36.                  | -                        | -                        | 2.5                      | 0.5                      | 85.4 <sup>b</sup> | 20.2 ± 0.3 <sup>b</sup> |
| 37.                  | -                        | -                        | 3.0                      | 0.5                      | 65.5 <sup>f</sup> | 14.4 ± 0.8 <sup>c</sup> |
| Analysis of variance |                          |                          |                          |                          |                   | F= 19,828               |
|                      |                          |                          |                          |                          |                   | d.f.= 11,510            |

Values are mean ± SE in a column followed by a common letter are not significantly different at the 5% level by DMRT.

Table (2): Rhizogenesis of *V. negundo* regenerated shoots after 2 weeks of culture.

| S.no.                | Plant Growth Regulator<br>IBA (mgL <sup>-1</sup> ) | Invitro rhizogenesis |                         |                        |
|----------------------|----------------------------------------------------|----------------------|-------------------------|------------------------|
|                      |                                                    | Root induction (%)   | Root no./ shoot         | Root length (cm)       |
| 1.                   | 0.25                                               | 85.7 <sup>c</sup>    | 8.4 ± 0.4 <sup>c</sup>  | 3.2 ± 0.5 <sup>c</sup> |
| 2.                   | 0.50                                               | 90.4 <sup>b</sup>    | 10.0 ± 0.2 <sup>b</sup> | 4.5 ± 0.7 <sup>b</sup> |
| 3.                   | 0.75                                               | 100.0 <sup>a</sup>   | 12.8 ± 0.1 <sup>a</sup> | 4.9 ± 0.5 <sup>a</sup> |
| 4.                   | 1.00                                               | 85.0 <sup>c</sup>    | 10.2 ± 0.6 <sup>b</sup> | 4.0 ± 0.1 <sup>b</sup> |
| 5.                   | 1.25                                               | 80.6 <sup>d</sup>    | 7.6 ± 0.3 <sup>d</sup>  | 3.7 ± 0.6 <sup>c</sup> |
| 6.                   | 1.50                                               | 77.2 <sup>c</sup>    | 5.5 ± 0.8 <sup>c</sup>  | 2.8 ± 0.2 <sup>d</sup> |
| Analysis of variance |                                                    |                      | F= 9.436                | F= 1.988               |
|                      |                                                    |                      | d.f. = 3,182            | d.f. = 1,876           |

Values are mean ± SE in a column followed by a common letter are not significantly different at the 5% level by DMRT.



Figure (1). In vitro differentiation and plant regeneration from nodal explants of *V. negundo*: a) shoot initials, b & c) multiple shoots plant

#### 4. DISCUSSION

This investigation reveals the importance of optimizing plant growth regulator combinations for efficient and rapid micropropagation of *V. negundo*. The failure of nodal explants to initiate shoots on hormone-free MS medium and the subsequent improvement after cytokinin supplementation indicate that exogenous cytokinins are important for activating axillary shoot development [6]. TDZ was superior to BAP and KIN for shoot induction in this study. The highest response obtained with  $2.0 \text{ mg L}^{-1}$  TDZ is consistent with earlier reports on *V. negundo*, in which TDZ was found to be an effective cytokinin for axillary shoot proliferation [13]. Kumar et al. [8], Iqbal et al. [12], Poearim et al. [14], and Shweta et al. [15] also observed successful shoot multiplication using TDZ alone or in combination with auxins on the in vitro response of *Vitex negundo* L.

The present study further demonstrated that combining cytokinin with a suitable auxin enhanced shoot regeneration. The TDZ+IAA combination produced a high frequency of shoot induction and a comparatively large number of shoots per explant. Such synergistic effects between cytokinins and auxins have been reported in several plant species and have also been documented for *V. negundo* [7,16]. The superior rooting response obtained using IBA is also consistent with previous micropropagation studies. In this experiment, half-strength MS medium containing  $0.75 \text{ mg L}^{-1}$  IBA produced the highest rooting frequency and favorable root development [17]. Reduction of basal salt concentration during rooting may have favored rhizogenesis in the regenerated shoots [2]. Successful acclimatization is a critical step in practical micropropagation because plantlets raised under high-humidity, low-stress in vitro conditions must gradually adapt to ex vitro environmental conditions. The complete survival reported in this study demonstrates the feasibility of transferring the regenerated *V. negundo* plantlets to field conditions [18]. The optimized regeneration system therefore provides a useful platform for rapid multiplication and ex situ conservation of *V. negundo*. It may also serve as a source of uniform planting material for further studies involving phytochemical production, medicinal evaluation, and conservation of elite germplasm.

#### 5. CONCLUSION

An efficient in vitro regeneration protocol was established for *Vitex negundo* using nodal explants via direct organogenesis. Among the cytokinins examined, TDZ was the most effective for shoot induction, with  $2.0 \text{ mg L}^{-1}$  TDZ producing 92.6% shoot induction. Further optimization with auxin indole-3-acetic acid (IAA) demonstrated that  $2.0 \text{ mg L}^{-1}$  TDZ +  $0.5 \text{ mg L}^{-1}$  IAA was highly effective for shoot multiplication, producing up to 96.3% shoot induction and 26.6 shoots per explant. For rooting, half-strength MS medium containing  $0.75 \text{ mg L}^{-1}$  IBA produced 100% root induction with 12.8 roots per shoot and approximately 4.9 cm mean root length. The regenerated plantlets were successfully acclimatized and showed 100% survival under the reported field conditions. The developed protocol can therefore be considered a promising approach for rapid multiplication and ex situ conservation of *V. negundo*. It may contribute to the availability of quality planting material and support the conservation of this economically and medicinally important plant.

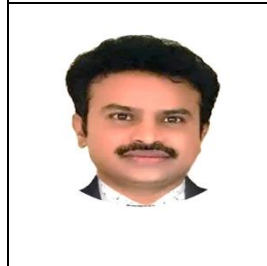
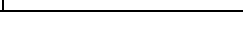
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## BIOGRAPHIES OF AUTHORS

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