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Rapid Multiplication of *Nyctanthes arbor - tristis* L. Through *In vitro* Axillary Shoot Proliferation

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Abstract: An efficient, rapid and reproducible plant regeneration protocol was successfully developed for *Nyctanthes arbor - tristis* using cotyledonary node explants excised from 15 day old aseptic seedlings. Of the two cytokinins, thidiazuron (TDZ) and 6-benzyladenine (BA) evaluated as supplements to Murashige and Skoog (MS) medium, TDZ at an optimal concentration of 1.0 μM whereas BA at 2.5 μM was found to be effective in the induction of maximum shoots. The combination of BA and α-naphthalene acetic acid (NAA) significantly enhanced the shoot multiplication. The regenerated shoots when subcultured on hormone free MS medium considerably increased the rate of shoot multiplication and shoot length by end of fourth subculture passage. *Ex vitro* rooting was achieved when the basal cut ends of regenerated shoots were dipped in 200 μM indole -3- butyric acid (IBA) for half an hour followed by transplantation in plastic pots containing sterile soilrite. The plantlets with well developed shoot and roots were successfully established in earthen pots containing garden soil and grown in greenhouse with 85% survival rate.

Key words: Cotyledonary node · *ex vitro* rooting · multiplication · *Nyctanthes arbor - tristis* · subculture

INTRODUCTION

Nyctanthes arbor - tristis L. (Night Jasmine) of the family Oleaceae is a small, sacred ornamental tree and planted in gardens almost throughout India for its fragrant flowers. The plant is used in traditional system of medicine for the treatment of chronic fever and rheumatism. It is also used as an anthelmintic and as a liver and nervine tonic. Leaves and seeds contains β-amyrin, β-sitosterol, nyctanthic acid, oleanolic acid, Iridoid and phenylpropanoid glycosides [1]. The Iridoid arbor tristoside A has been reported to have pronounced anticancer activity.

Micro propagation is one of the innovative methods of asexual propagation that has proved to be effective for *in vitro* propagation of medicinal and aromatic plants and in commercial exploitation of valuable plant derived pharmaceuticals [2-7]. *In vitro* plant regeneration is also the most important step for successful implementation of various biotechnological techniques used for plant improvement programmes. There is no previous report on *in vitro* studies in *N. arbor - tristis*. Therefore, the objective of the present study was to develop a rapid and reproducible *in vitro* regeneration system from cotyledonary node explants through high frequency shoot proliferation and growth, followed by successful *ex vitro* establishment of regenerated plants.

MATERIAL AND METHODS

Establishment of aseptic seedlings: Seeds of *N. arbor - tristis* were collected from the Botanical Garden of the University and washed thoroughly under running tap water for 30 min to remove adherent particles, then treated with a liquid detergent labolene (5% v/v) for 20 min followed by washing in tap water and rinsed five times with double distilled water. These were surface disinfected for 4 min in 0.1% (w/v) $HgCl_2$ and finally rinsed five times with sterile double - distilled water. The seeds were inoculated in MS medium [8] for germination. Cotyledonary node excised from 15 day old aseptic seedlings were used as explants.

Culture media and conditions: MS medium supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar was used during the investigation. The pH of the medium was adjusted to 5.8 by 1N NaOH. The culture vials containing the media were autoclaved at 121°C at 1.06 kg cm^{-2} for

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Table 1: Effect of different plant growth regulators on shoot regeneration from cotyledonary node explants of *N. arbor - tristis* after 8 weeks of culture

Plant growth regulators (μM)					
TDZ	BA	NAA	% Regeneration	Number of shoots/explant	Shoot length (cm)
0.5			85	9.8±0.38^{bc}	2.0±0.17^{ef}
1.0			90	13.6±0.90^{a}	4.4±0.26^{a}
2.5			80	10.5±0.61^{b}	3.5±0.23^{bc}
5.0			74	8.6±0.37^{cde}	3.2±0.17^{bcd}
10.0			67	7.0±0.40^{efg}	2.0±0.23^{ef}
	0.5		70	5.6±0.41^{g}	1.6±0.32^{f}
	1.0		77	8.7±0.43^{cd}	2.8±0.29^{cd}
	2.5		84	11.4±0.74^{b}	3.8±0.34^{ab}
	5.0		72	7.6±0.40^{def}	2.6±0.23^{de}
	10.0		63	6.0±0.34^{fg}	1.4±0.17^{f}
1.0		0.1	92	10.0±0.69^{cd}	3.8±0.28^{bc}
1.0		0.5	95	13.0±0.83^{ab}	4.6±0.31^{ab}
1.0		1.0	88	8.5±0.46^{d}	3.5±0.23^{c}
	2.5	0.1	87	12.1±0.72^{bc}	4.4±0.26^{ab}
	2.5	0.5	93	15.1±0.95^{a}	5.2±0.34^{a}
	2.5	1.0	82	10.9±0.67^{bcd}	3.9±0.23^{bc}

Values represent means±SE. Means followed by the same letter within columns are not significantly different (p=0.05) using Duncan's multiple range test

Table 2: The evaluation of morphogenetic potential of shoot culture obtained from TDZ (1.0 μM) after being tested for five subculture passages on growth regulator free MS medium

Subculture Passages	Number of shoots/explant	Shoot length (cm)
1st	13.6±0.88^{c}	4.4±0.23^{c}
2nd	17.9±0.78^{b}	5.6±0.30^{b}
3rd	20.6±1.06^{ab}	6.1±0.29^{ab}
4th	22.4±1.53^{a}	6.6±0.35^{a}
5th	22.4±1.53^{a}	6.6±0.35^{a}

Values represent means±SE. Means followed by the same letter within columns are not significantly different (p=0.05) using Duncan's multiple range test

20 min. All the cultures were maintained at 24±2°C under 16 h photoperiod with a photosynthetic photon flux density (PPFD) of 50 μmol $m^{-2}s^{-1}$ provided by cool white fluorescent lamps (Phillips, India) and with 60-65% relative humidity.

Shoot induction and multiplication: For multiple shoot induction, cotyledonary node explants were placed on MS medium supplemented with various cytokinins (TDZ and BA) at different concentrations (0.5, 1.0, 2.5, 5.0 and 10.0 μM) either singly or in combination with NAA (0.1, 0.5 and 1.0 μM). Subsequent subcultures were at 2 weeks intervals onto the same fresh media. The shoots induced from TDZ either singly or in combination with NAA were transferred after four weeks to MS medium devoid of plant growth regulators. The multiplication rate was tested up to fifth passage and total number of shoots and shoot length was recorded at each passage of subculturing. The maximum percent regeneration, number of shoots and shoot length were recorded after 8 weeks of culture.

***Ex vitro* root formation and acclimatization:** For *ex vitro* root induction, excised shoots (3-5 cm) with four or more leaves were harvested from each subculture and their basal portion were dipped in different concentrations of IBA (100, 200, 300 and 500 μM) for half an hour and then planted in plastic pots containing sterile soilrite under diffuse light (16/8 h photoperiod) conditions. Potted plantlets were covered with transparent polythene bags to ensure high humidity and watered every 3 days with half strength MS salt solution for 2 weeks. Polythene bags were opened after 2 weeks in order to acclimatize plants to field conditions. Data were recorded on percentage of rooting, number and length of roots after 4 weeks of *ex vitro* transplantation.

Statistical analysis: All the experiments were conducted with a minimum of 20 replicates per treatment. The experiments were repeated three times. The data was analyzed statistically using SPSS Ver. 10 (SPSS Inc., Chicago, USA). The significance of differences among means was carried out using Duncan's multiple range test at p=0.05. The results are expressed as the means±SE of three experiments.

RESULTS AND DISCUSSION

The morphogenetic responses of cotyledonary node explants to TDZ and BA either alone or in combination with NAA are summarized in Tables 1 and 2. Explants cultured on MS medium without cytokinins failed to produce shoots even after 4 weeks. MS medium supplemented with different concentrations of TDZ and BA (0.5-10.0 μM), resulted in the induction of multiple

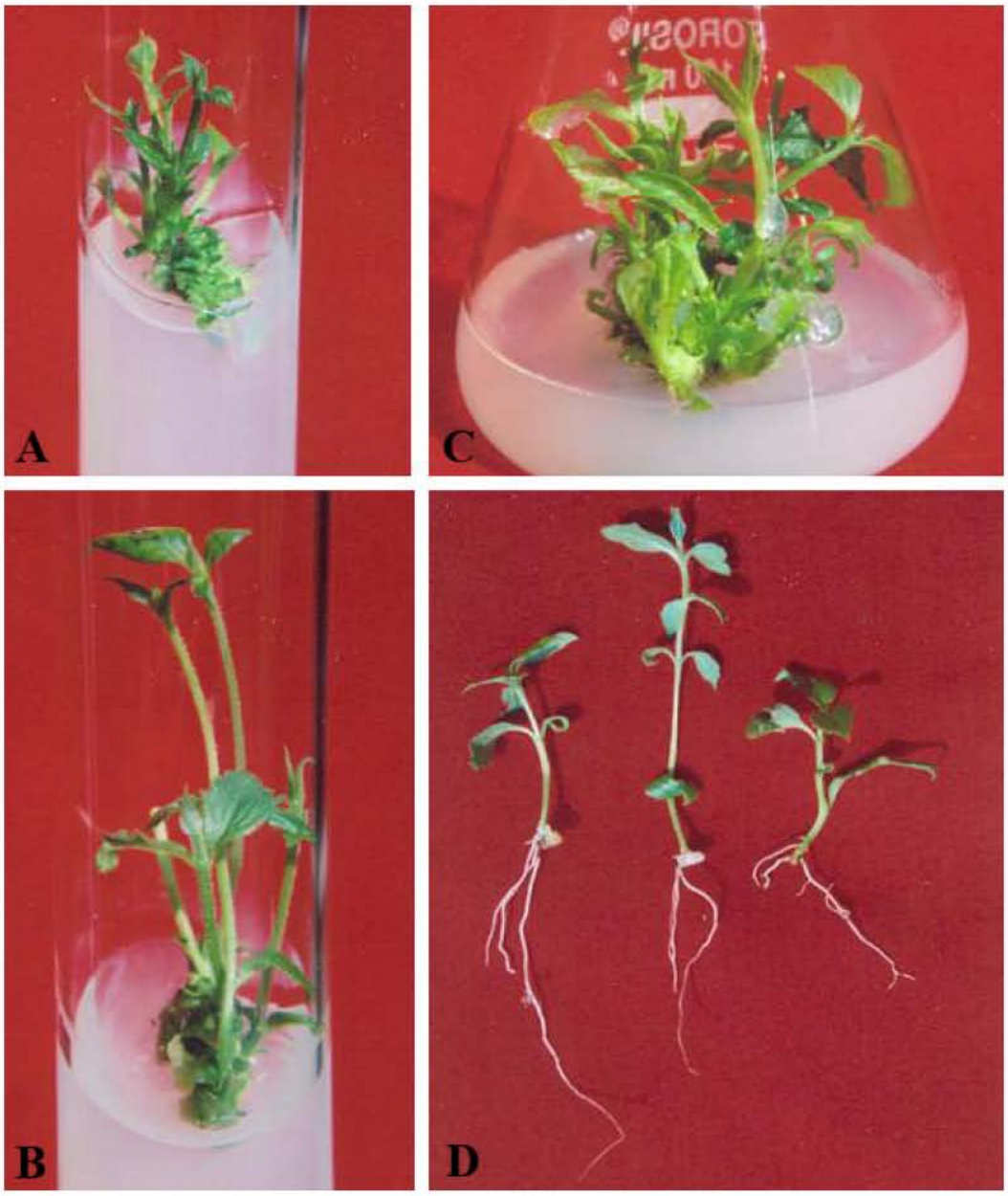

Fig. 1 A-D: *In vitro* regeneration and plant establishment of *N. arbor - tristis*. (A) Multiple shoot induction on MS + TDZ (1.0 µM), (B) Four -week -old culture showing well developed leaves on growth regulator free MS medium, (C) Proliferation of shoots on growth regulator free MS medium after eight weeks, (D) *Ex vitro* rooted plantlets

Table 3: The efficiency of root induction from regenerated shoots of *N. arbor - tristis* dipped in IBA solution for half an hour after 4 weeks of transplantation

IBA (μM)	% Rooting	Number of roots/shoot	Root length (cm)
100	50	4.5±0.31^{b}	1.9±0.23^{c}
200	75	7.3±0.37^{a}	3.9±0.29^{a}
300	60	4.3±0.29^{b}	3.2±0.26^{ab}
500	45	3.1±0.32^{c}	2.5±0.31^{bc}

Values represent means±SE. Means followed by the same letter within columns are not significantly different (p=0.05) using Duncan's multiple range test

shoots from cotyledonary node explants after 2 weeks of incubation.

All the concentrations of TDZ and BA facilitated shoot bud differentiation, but TDZ being more efficient than BA in terms of percent regeneration, number of shoots and shoot length. Among the various concentrations of TDZ and BA tested, 1.0 μM TDZ showed the highest shoot regeneration frequency (90%), number of shoots (13.6±0.90) and shoot length (4.4±0.26) (Fig. 1A). However, BA was found to be effective at 2.5 μM in inducing maximum number of shoots (11.4±0.74) in 84% cultures (Table 1).

The effectiveness of TDZ in shoot bud differentiation has been documented in number of plants [9-13]. The regeneration frequencies and number of shoots declined with an increase in cytokinin concentration beyond the optimal level. Reduction in the number of shoots in all the concentrations higher than optimal level has also been reported for several plants [14-16].

The synergistic influence of auxin with cytokinins was evident when optimal concentration of TDZ and BA were tested with different concentrations of NAA (0.1, 0.5 and 1.0 μM) (Table 1). BA in combination with NAA markedly enhanced the percent regeneration, number of shoots and shoot length whereas TDZ and NAA combination did not improve the parameters evaluated. Among all the cytokinins - auxin combinations tried, the maximum percent regeneration (93%), number of shoots (15.1±0.95) and shoot length (5.2±0.34) per explant were obtained at BA (2.5 μM) and NAA (0.5 μM). This was considered the optimal growth regulator combination for shoot regeneration in *N. arbor - tristis* among all the treatments. The role of auxin incorporated in the medium in combination with cytokinins for shoot multiplication has been reported in number of cases [17-19].

The effect of subculture passages was also evaluated on shoot multiplication in MS medium supplemented with TDZ (1.0 μM) after transfer to MS basal medium without TDZ. The cultures grown continuously on TDZ containing media formed fasciated and distorted shoots. The deleterious effect of continued presence of TDZ on the growth and multiplication of Chickpea, *Rauvolfia tetraphylla* and *Capsicum annuum* [6, 20, 21] has been reported. The highest rate of shoots (22.4±1.53) and shoot length (6.6±0.35) per explant increased during the first four culture passages, got stabilized at fifth passage (Fig. 1B & C) (Table 2) and beyond which a gradual decline in multiplication rate was noticed (data not shown). In *Aegle marmelos*, frequency of shoot proliferation and growth of shoot continued through five subculture passages without any sign of decline [22] whereas in *Bacopa monniera*, an increase in shoot induction and multiplication has been reported upto third subculture passage beyond which the frequency and number of shoots decreased [12].

Rooting was carried out by *ex vitro* method. The basal portion of regenerated shoots were dipped in different concentrations of IBA (100-500 μM) for half an hour and subsequently planted in plastic pots containing sterile soilrite (Table 3). Best results were recorded when shoots were dipped in IBA (200 μM) as it gave the maximum frequency (75%), number of roots (7.3±0.37) and root length (3.9±0.29) (Fig. 1D). *Ex vitro* rooting was also found to be effective in *Tylophora indica* and in *Capsicum annuum* [23, 24]. Rooted plantlets were transferred to earthen pots containing garden soil and organic manure (3: 1) and kept in greenhouse for acclimatization. After one month of transfer to soil, 85% of plants survived in the greenhouse. There was no detectable variation among the potted plants with respect to morphology and growth characteristics.

The present study describes for the first time an efficient method for *in vitro* regeneration of *N. arbor - tristis*. This protocol is successful and could be used for large scale multiplication and propagation of this important woody ornamental /medicinal tree.

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