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ASSESSMENT OF FACTORS AFFECTING MICROPROPAGATION AND *EX VITRO* ACCLIMATIZATION OF *NYCTANTHES ARBOR-TRISTIS* L.

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Rapid differentiation of multiple shoots was observed in 94% of nodal explants of one year old *Nyctanthes arbor-tristis* L. plants. Shoot bud induction and multiplication took place on Murashige and Skoog (MS) medium supplemented with two cytokinins, i.e. Benzyladenine (BA) or Kinetin (Kn) either alone or in combination with different auxins, indole-3-butyric acid (IBA), indole-3-acetic acid (IAA) or α-naphthalene acetic acid (NAA). Between different media, pH levels and growth regulators tried, the optimum condition for maximum regenerative response was obtained on MS+Kn (2.5 μM)+NAA (0.5 μM) media at 5.8 pH, forming cultures with 23.26±0.89 number of shoots and 6.36±0.80 cm shoot length after 8 weeks of culture. Histological sections confirmed the formation of multiple buds from nodal explants. Rooting was achieved *ex vitro* by dipping the basal ends of microshoots in 200 μM IBA for 30 min followed by their transplantation in sterile soilrite. The plantlets with well-developed shoot and root system were successfully established in garden soil and grown outside in a greenhouse with a 80% survival rate.

Keywords: Clonal propagation – *ex vitro* rooting – nodal segment – *Nyctanthes arbor-tristis* – Oleaceae

INTRODUCTION

Nyctanthes arbor-tristis (Oleaceae), commonly known as harsinghar is a small evergreen ornamental tree found throughout India and is highly valued for its medicinal properties. The leaves and bark extracts are useful as laxative, diaphoretic, diuretic, anthelmintic and expectorant [4] and can be employed for the treatment of several diseases like asthma, rheumatism, sciatica, dyspepsia, chronic fever, cough, inflammations, constipation, baldness, premature greying of hair and various nervous disorders. A perusal of literature reveals that plant contains useful secondary metabolites like nyctanthic acid, friedlin, β-sitosterol, oleanolic acid and iridoid arbor-tristoside-A isolated from leaves and whole plant extracts, that have pronounced antidiabetic and anticancer activities [17, 25]. The species is traditionally propagated by seeds,

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Abbreviations: BA – 6-Benzyladenine; IAA – Indole-3-acetic acid; IBA – Indole-3-butyric acid; Kn – Kinetin (6-furfurylaminopurine); NAA – α-naphthalene acetic acid.

however, the germination problem of seeds has not yet been solved as some phenolic compounds leached out of imbibed seeds interfere in the germination process and death of many young seedlings occur under natural conditions [6, 35]. Due to destruction of its natural habitat, excessive over exploitation and unresolved inherent problems of seed viability and germination, the natural strands of this priority plant has been markedly depleted [30].

Tissue culture methods are widely applied for conservation and mass propagation of a number of medicinal plants [1, 3, 11, 12, 18]. Earlier, only few attempts on *in vitro* regeneration and propagation were made on *Nyctanthes arbor-tristis* [26, 29, 30, 34] albeit to a limited extent. Recent reports of Rout et al. [29, 30] do not provide sufficient satisfactory data regarding the number of shoots (6.65 shoots per explant) therefore, considerable efforts are still needed to improve the rate of multiplication. The objective of the present study was to standardize a reproducible protocol for *in vitro* propagation through high frequency axillary shoot proliferation from nodal buds of *Nyctanthes arbor-tristis* L.

MATERIALS AND METHODS

Plant material

Young shoots collected from one year old plant maintained at the botanical garden of the University, were treated firstly with a fungicide Bavistin (0.1% w/v) for 10 min followed by washing under running tap water for about half an hour. Thereafter, the shoots were disinfected with a liquid detergent, Labolene 5% (v/v) (Qualigens, India) for 15 min followed by four to six washing with sterilized double distilled water to make the plant material free from detergent. These were then surface sterilized with 0.1% (w/v) $HgCl_2$ solution for about 5 min followed by six times repeated washes with sterilized double distilled water. The nodal segments were excised aseptically and used as an explant. Disinfection process was carried out under aseptic conditions in a laminar air flow cabinet.

Culture medium and culture conditions

The culture media tested were Murashige and Skoog (MS 1962) [23], Phillips and Collins (L_2 1979) [27], Lloyd and McCown (WPM 1980) [22] and Gamborg's (B_5 1968) [13] with 3% (w/v) sucrose and 0.8% (w/v) agar. Plant growth regulators and their combinations were added to the medium as specified below. The pH of the media was adjusted at 5.8 with 1N NaOH or HCl prior to autoclaving at 121 °C (1.06 kg cm^{-2}) for 20 min. All cultures were maintained at 24 ± 2 °C, 16 h photoperiod with a photosynthetic photon flux density (PPFD) of 50 µmol m^{-2} s^{-1} provided by cool white fluorescent lamps (Philips, India) with 60 ± 5% relative humidity (RH).

Shoot induction and multiplication

Nodal segments cultured on MS (1962) medium were supplemented with cytokinins either alone (BA or Kn at 0.5, 1.0, 2.5, 5.0 and 10.0 μM) or in combination with auxins (IBA, IAA or NAA) at various concentrations (0.1, 0.5 and 1.0 μM). Different basal media were tested with Kn (2.5 μM) and NAA (0.5 μM) combination to detect the best suited media for multiplication. The effect of different pH of the medium (5.0, 5.4, 5.8, 6.2 and 6.6) was also examined with the same hormonal combination in MS medium. All cultures were transferred to fresh medium after every 2 weeks. The percentage of explant producing shoots, number of differentiated shoots and shoot length were recorded after 8 weeks of culture.

Ex vitro *rooting and acclimatization*

For *ex vitro* rooting, individual microshoots of about 3–5 cm with three or more leaves were harvested from the shoot clusters and their basal portion were dipped into different concentrations of IBA (100, 150, 200, 250 and 300 μM) for half an hour and planted in thermocups containing sterile soilrite under diffuse light conditions (16/8 h photoperiod). Potted plantlets were covered with transparent polythene bags to ensure high humidity and irrigated after every 3 days with half strength MS salt solution for two weeks. Bags were opened after 2 weeks in order to acclimatize the plant to field conditions. Data were recorded on percentage rooting, number of roots and root length after 4 weeks of *ex vitro* transplantation.

Histological analysis

To confirm the regeneration of multiple shoot buds from the nodal explants, histological examination of explants was performed after 15 days. Tissues were fixed in formalin : glacial acetic acid : ethanol, 4 : 6 : 90 (v/v) solution. Fixed tissues were dehydrated through an ethanol/xylol series and embedded in paraffin wax (60 °C). Serial sections 10 μM thickness were cut using a Spencer 820 microtome (American Optical Corp., Buffalo, NY, USA) and the resulting paraffin ribbons were passed through a series of deparaffinising solutions and stained in saffranin and fast green solutions. The sections were examined under an optical microscope (CH20i, Olympus, Tokyo, Japan).

Statistical analysis

All experiments were conducted with a minimum of 10 replicates per treatment and repeated three times. The data was analyzed statistically using SPSS ver. 16 (SPSS Inc., Chicago, USA). The significance of differences among means was carried out using Duncan's multiple range test (DMRT) at $P = 0.05$. The results are expressed as means ± SE.

RESULTS AND DISCUSSION

Shoot induction and proliferation

Effect of cytokinins and auxins combination

The axillary bud break could not be achieved on all the basal medium tested without growth regulators. From experiments comprising a large array of treatments employing cytokinins and auxins either singly or in different combinations at different concentrations, a distinct variation was noticed in the regeneration percentage, number of shoots and shoot length. Between the two cytokinins (BA or Kn) tested, kinetin treated explants were the most responsive, exhibiting the higher rate of shoot regeneration accompanied with the maximum number of shoots (Table 1). Swelling followed by bud-break of dormant axillary buds and their differentiation into shoot buds took place after 15 days of culture. The histological sections revealed direct differentiations of multiple shoot buds from the nodal explants (Fig. 2A, B, see arrows). The data given in Table 1 revealed that the highest number of shoots (16.43 ± 0.80) accompanied with the highest shoot regeneration frequency (90 %) was obtained at 2.5 μM Kn. However, a lower number of shoots (13.56 ± 0.72) were produced with same concentrations of BA (2.5 μM). In our previous experiment [34], the phenyl urea derived cytokinin, TDZ was more effective in enhancing multiple shoots from cotyledonary node explants. This may be influenced by the plant growth regulator to explant type interaction. But in present case, Kn was more effective than any other

Table 1
Effect of various concentrations of different cytokinins on shoot regeneration from mature nodal explants of *N. arbor-tristis* after 8 weeks of culture

Plant growth regulators, μM		% Regeneration	Number of shoots/ explants	Shoot length (cm)
BA	Kn			
0.5		53	4.83 ± 0.66^{fg}	1.13 ± 0.23^{e}
1.0		67	10.53 ± 0.95^{cd}	3.90 ± 0.40^{b}
2.5		80	13.56 ± 0.72^{b}	4.50 ± 0.65^{ab}
5.0		60	8.23 ± 1.17^{de}	3.03 ± 0.20^{bcd}
10.0		40	2.56 ± 0.69^{g}	1.60 ± 0.30^{de}
	0.5	60	11.90 ± 0.95^{bc}	2.20 ± 0.36^{bc}
	1.0	87	14.10 ± 0.58^{ab}	3.60 ± 0.26^{bc}
	2.5	90	16.43 ± 0.80^{a}	5.40 ± 0.83^{a}
	5.0	70	13.53 ± 0.86^{b}	3.10 ± 0.58^{bcd}
	10.0	50	6.10 ± 0.49^{ef}	3.30 ± 0.51^{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.

Fig. 1. In vitro regeneration and plant establishment of *Nyctanthes arbor-tristis*. A – Induction of shoots on MS + Kn (2.5 µM) + NAA (0.5 µM) after 4 weeks of culture. B – Proliferation and multiplication of shoots on MS + Kn (2.5 µM) + NAA (0.5 µM) after 8 weeks of culture. C – An *ex vitro* rooted plantlet. D – An acclimatized plant in sterile soilrite

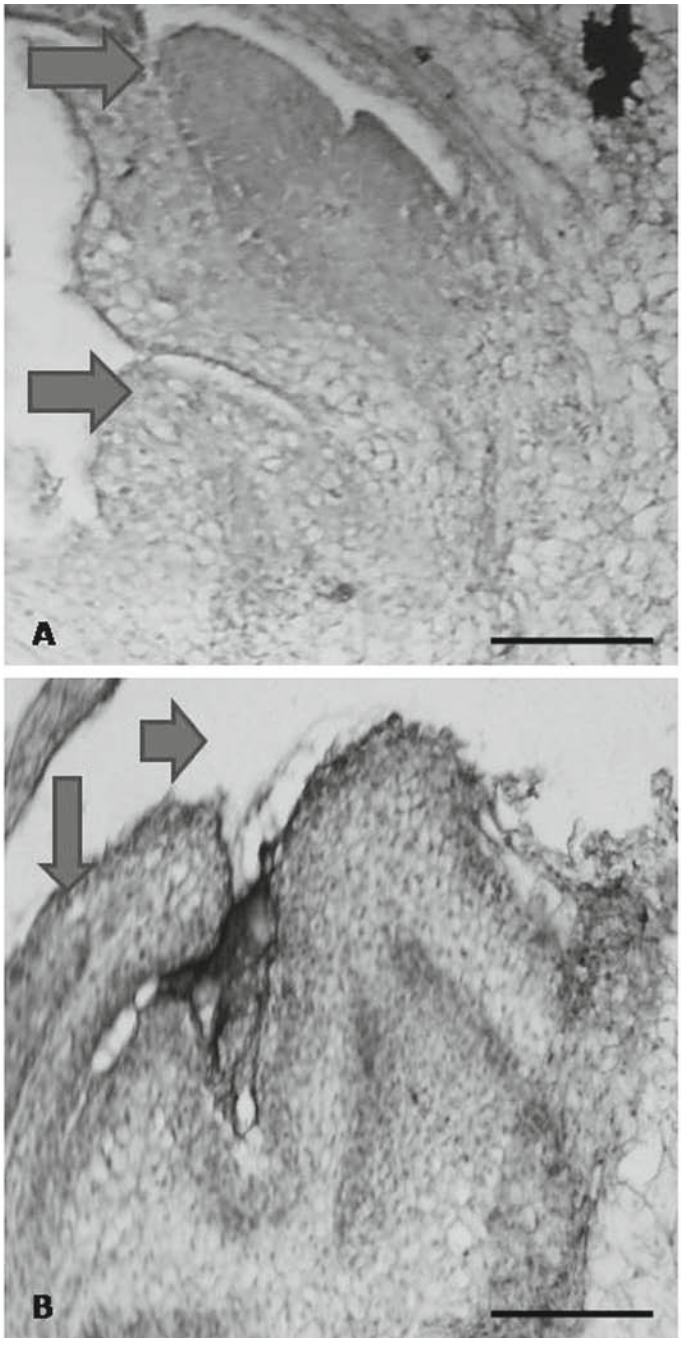

Fig. 2. Longitudinal sections showing direct differentiation of shoots buds from the nodal segments of *N. arbor-tristis* L. Scale bar = 100 µm

Table 2
Effect of various concentrations of different auxins in combination with optimal concentration of BA or Kn (2.5 μM) on shoot regeneration from nodal explants after 8 weeks of culture

Plant growth regulators (μM)					% Regeneration	Number of shoots/explants	Shoot length (cm)
BA	Kn	IBA	IAA	NAA			
2.5		0.1			57	9.63 ± 0.88^{j}	2.03 ± 0.20^{ghi}
2.5		0.5			66	13.60 ± 0.47^{fgh}	3.66 ± 0.37^{def}
2.5		1.0			50	7.10 ± 0.66^{k}	$l2.60 \pm 0.30^{fgh}$
2.5			0.1		53	5.66 ± 0.37^{l}	1.00 ± 0.00^{i}
2.5			0.5		57	7.00 ± 0.57^{kl}	1.43 ± 0.23^{i}
2.5			1.0		43	5.16 ± 0.68^{l}	1.20 ± 0.20^{c}
2.5				0.1	80	17.33 ± 0.37^{de}	5.10 ± 0.23^{bc}
2.5				0.5	90	18.53 ± 0.90^{cd}	5.80 ± 0.41^{ab}
2.5				1.0	77	15.53 ± 0.56^{ef}	4.16 ± 0.32^{cde}
	2.5	0.1			60	12.50 ± 0.92^{gh}	3.43 ± 0.29^{def}
	2.5	0.5			70	14.63 ± 0.57^{fg}	4.50 ± 0.28^{cd}
	2.5	1.0			66	12.00 ± 1.36^{hi}	3.03 ± 0.31^{efg}
	2.5		0.1		53	8.73 ± 0.64^{jk}	3.10 ± 0.26^{efg}
	2.5		0.5		60	10.10 ± 0.66^{ij}	3.83 ± 0.23^{de}
	2.5		1.0		40	8.33 ± 0.88^{jk}	1.86 ± 0.34^{hi}
	2.5			0.1	90	21.13 ± 0.86^{ab}	5.70 ± 0.68^{ab}
	2.5			0.5	94	23.26 ± 0.89^{a}	6.36 ± 0.80^{a}
	2.5			1.0	83	20.00 ± 0.75^{bc}	5.16 ± 0.35^{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.

cytokinins. Reduction in the parameters was noticed at higher or lower concentrations of cytokinins used beyond the optimum level. In the present endeavour, it was evident that the presence of a cytokinin was found to be necessary in axillary bud multiplication from nodal cuttings. Our results are in accordance with the studies made on *Limonium cavenillesii* [2], *Alpinia galanga* [7], *Bauhinia vahlii* [10], *Eurycoma longifolia* [16] and *Allium cepa* [19] where Kn was the most effective cytokinin in shoot multiplication.

Addition of different auxins (IBA, IAA or NAA) at different concentrations (0.1, 0.5 and 1.0 μM) along with optimal concentration of BA (2.5 μM) or Kn (2.5 μM) was also assessed with a view to improve the response and the results obtained proved beneficial in the parameters evaluated. In the present investigation, a combination of NAA and Kn gave a triggering synergistic response with regard to shoot bud induction and regeneration frequency of explants. A maximum of 23.26 ± 0.89 shoots per explants were differentiated with 6.36 ± 0.80 cm shoot length on MS medium

augmented with Kn (2.5 μM) and NAA (0.5 μM) with 94% regenerative potentiality within 8 weeks of culture (Table 2) (Fig. 1A, B). The addition of auxins to the optimal concentration of Kn or BA significantly increased the frequency of shoot differentiation when compared to Kn or BA alone in the present study. Many studies related to *in vitro* organogenesis underlines the importance of auxin/cytokinin ratio in the culture medium. This specific combination (Kn-NAA) has been reported successful in

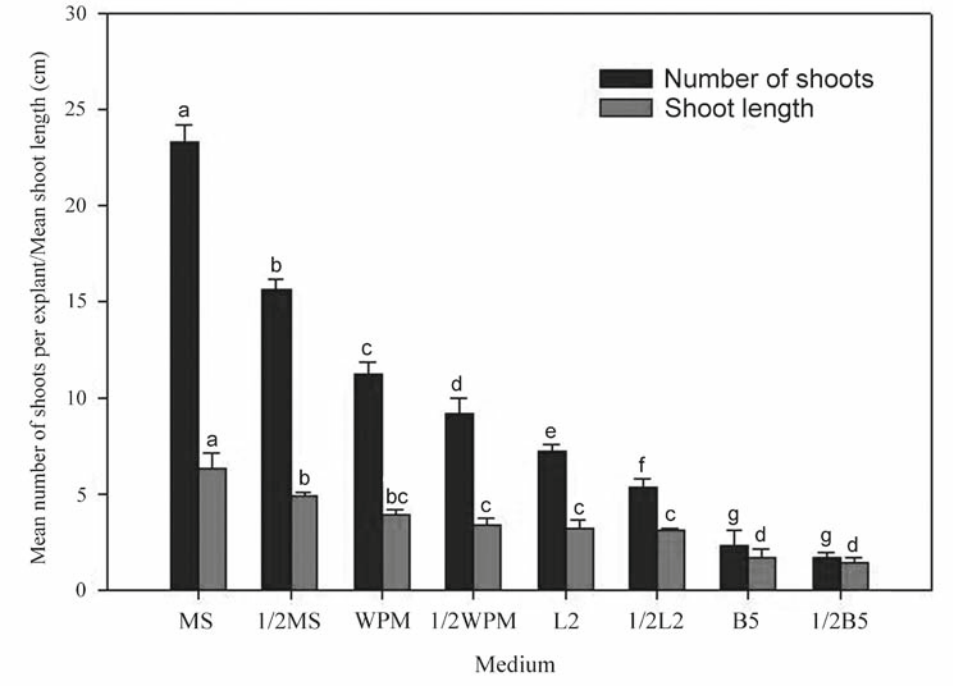

Fig. 3. Effect of different medium on shoot regeneration from nodal explants of *N. arbor-tristis* L. supplemented with Kn (2.5 μM) + NAA (0.5 μM) after 8 weeks of culture. The bars represent mean ± SE. Bars denoted by the same letter within response variables are not significantly different (P = 0.05) using Duncan's multiple range test

multiplying a number of plant species including *Myrica esculenta* [5, 25], *Cordia verbenacea* [21], *Alpinia officinarum* [31] and *Cephalis ipecacuanha* [9]. Based on this information, the present study also exemplifies the positive modification of multiplication efficacy by low concentration of an auxin with an optimal concentration of a cytokinin. For further multiplication process, cultures were repeatedly subcultured onto the same regenerating medium after every two weeks. During the subculture passages, the number of shoots increased without any decline in shoots number (data not shown). A similar subculturing effect was also reported by Siddique and Anis [32, 33] in *Cassia angustifolia* and *Balanites aegyptiaca.*

Effect of different media

Different basal media, i.e. MS; ½ MS; B_5; ½ B_5; L_2; ½ L_2; WPM and ½ WPM with optimal concentration of Kn (2.5 µM) and NAA (0.5 µM) were examined for inducing maximum shoot multiplication and shoot length. Of the various strength of the different basal media tried, full strength MS medium followed by ½ MS and WPM medium were found suitable for maximum shoot induction and proliferation (Fig. 3). Similar studies were also reported by Nandwani [25] and Faisal et al. [12]. Furthermore, L_2 and B_5 gave satisfactory results.

Effect of different pH

The pH affects nutrient uptake as well as enzymatic and hormonal activities in plants. The optimal pH regulates the cytoplasmic activity that affects the cell division and growth of shoots and it does not interrupt the function of the cell membrane and the buffered pH of the cytoplasm [8]. The detrimental effects of adverse pH are generally related to an imbalance in nutrient uptake rather than to direct cell damage [15]. In the present study, healthy shoots with fully expanded leaves developed at 5.8 pH (Fig. 4). Similar findings where the rate of shoot proliferation significantly increased

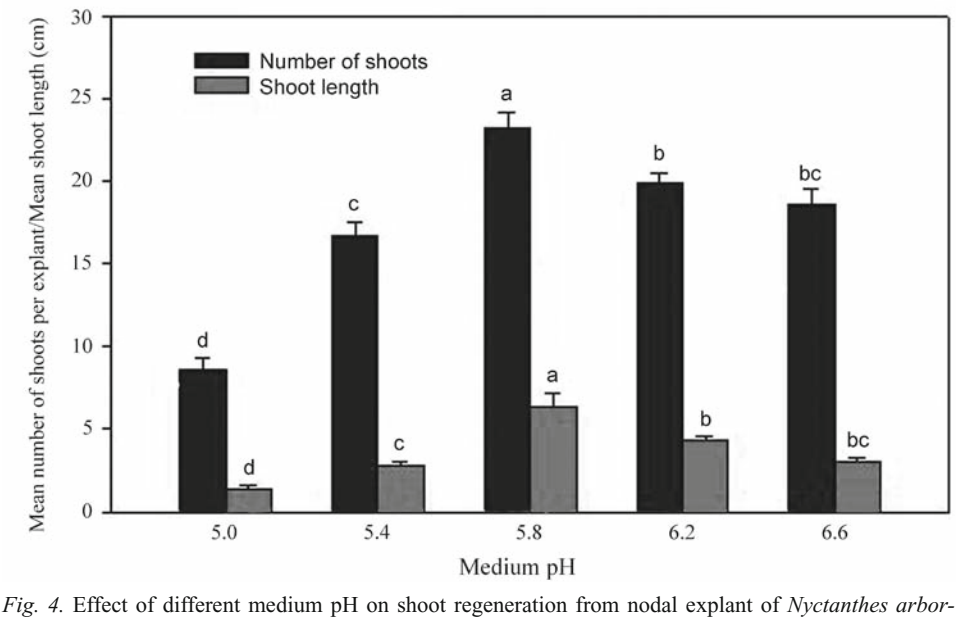

Fig. 4. Effect of different medium pH on shoot regeneration from nodal explant of *Nyctanthes arbor-tristis* augmented with Kn (2.5 µM) + NAA (0.5 µM) after 8 weeks of culture. The bars represent mean ± SE. Bars denoted by the same letter within response variables are not significantly different (P = 0.05) using Duncan's multiple range test

when pH was adjusted to 5.8 were found in *Azadirachta indica* [14], *Calophyllum apetalum* [24] and *Mucuna pruriens* [12]. Results deviate from the optimum level when the pH is either low or high. In addition to this, status of the solidifying agent in a medium is readily affected by pH.

Rooting and acclimatization

Healthy shoots (4–5 cm) were excised and transferred to the root inducing medium containing different strength of MS (full, ½ MS, ⅓ MS and ¼ MS) augmented with various auxins (IBA, IAA and NAA) but unfortunately no rooting was induced even after 4 weeks of incubation. Therefore, *ex vitro* rooting and acclimatization experiment was carried out which eliminates an *in vitro* rooting step. Isolated microshoots from shoot clusters were pulse treated for half an hour with different doses of IBA followed by their transplantation in sterile soilrite and acclimatized according to the procedure as explained in material and method. Adventitious root formation took place in a treatment of 200 µM IBA where a maximum of 8.96 ± 1.10 roots per shoot and 5.40 ± 0.61cm root length was observed (Table 3) (Fig. 1C). However, in other

Table 3
Effect of IBA on *ex vitro* root formation after 4 weeks

IBA (µM)	% Regeneration	No. of roots/shoot	Root length (cm)
00	00	0.00 ± 0.00^{e}	0.00 ± 0.00^{d}
100	55	1.00 ± 0.00^{d}	0.63 ± 0.18^{c}
150	75	3.83 ± 0.59^{c}	1.50 ± 0.28^{bc}
200	90	8.96 ± 1.10^{a}	5.40 ± 0.61^{a}
250	80	6.63 ± 0.71^{b}	4.66 ± 0.35^{a}
300	60	2.46 ± 0.49^{cd}	2.43 ± 0.57^{b}

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.

doses of IBA, the roots were thinner and weak. *Ex vitro* rooting is cost effective, less time consuming method and has been found to be effective in a number of plants like in *Achras sapota* [28], *Fraxinus pennsylvanica* [20], *Vitex negundo* [1], *Syringa vulgaris* [36] and *Malus zumi* [37]. Successfully established plantlets (Fig. 1D) were subsequently transferred to the field conditions, where 80% plantlets survived and continued to grow further.

CONCLUSIONS

Our findings are significant in obtaining maximum number of shoots than the earlier available protocols. The present method established for medicinally important plant, *N. arbor-tristis* will be helpful for obtaining high quality secondary metabolites to pharmaceutical industries and for further genetic transformation studies.

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