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RESEARCH ARTICLE

INFLUENCE OF PUTRESCINE ON *IN VITRO* SHOOT MORPHOGENESIS FROM SHOOT-TIP EXPLANTS OF *NYCTANTHES ARBOR-TRISTIS (*L.): AN IMPORTANT MEDICINAL PLANT

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ABSTRACT

Seed An improved and effective method for shoot organogenesis and plant regeneration in *Nyctanthes arbor-tristis* (L.) was established. Shoot-tip explants were cultured on Murashige and Skoog (MS) media fortified with different concentrations of benzyl amino purine (BAP), naphthalene acetic acid (NAA) and zeatin for efficient shoot induction. BAP at 1.0 mgl^{-1} was found most effective for initial shoot bud induction. About 76.67% explants responded, resulting in the highest (5.9 ±0.11) number of shoots per explant after 30 days of culture. A combination having 1.0 mgl^{-1} BAP with 0.3 mgl^{-1} NAA in MS medium, performed best as shoot multiplication medium with highest (10.33 ±0.09) number of shoots per explant with a mean shoot length of 3.81 ±0.09 cm after six weeks. For improving the shoot morphogenesis, different concentrations of putrescine (10-100 mgl^{-1}) were tested as an ethylene modulator. Putrescine at a concentration of 50 mgl^{-1} had maximum promoting influence on percentage of culture response (93.33%) and number of shoots (12.67 ±0.13) regenerated per explants with a mean shoot length of 6.84 ±0.13cm. The *in vitro* rooted plantlets were hardened and finally successfully transplanted in natural soil with almost 92% field survival rate.

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INTRODUCTION

Nyctanthes arbor-tristis (L.) commonly known as Harsinghar is an important medicinal and ornamental plant belonging to family Oleaceae. It is native to Southern Asia and distributed throughout India and its neighboring countries. It is a hardy large shrub or small tree having small, white flowers with bright orange corolla tubes. The fruit is a flat brown heart-shaped to round capsule with two sections each containing a single seed (Anonymous 2001). It demonstrates diverse pharmacological and biological activities like antibacterial, antioxidant, anti-inflammatory, antipyretic, antimalarial and antibiotic activities (Priya and Ganjewala 2007). Different parts of this plant are known to possess various medicinal properties and are used by rural mainly tribal people of India along with its use in Ayurveda, Sidha and Unani systems of medicines (Jahan 2011).

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Apart from these properties, an extract called nyctanthin has been used as dye. *N. arbor-tristis* is traditionally propagated by seeds and semi hardwood cuttings; however propagation through seed is unreliable due to poor germination and death of many seedlings under natural conditions (Anonymous 2001). The resurgence of public interest in plant based medicines coupled with rapid expansion of pharmaceutical industries necessitated an increased demand for the medicinal plant leading to over-exploitation. Due to destruction of its natural habitat, excessive over exploitation and unresolved problems of seed viability and poor germination, the natural stand of this important medicinal and ornamental plant have been markedly diminished (Rout *et al*., 2007; Jahan *et al*, 2011). For safeguarding this species from extinction, efforts need to be directed to propagate this plant using alternative approaches such as tissue culture techniques. Though there are some reports available on *in vitro* regeneration in *N. arbor-tristis* (Rout *et al*., 2007; Chawla *et al,* 2010; Jahan *et al*, 2011; Sahu *et al.,* 2012 and Bansal *et al*., 2012), the shoot regeneration and root induction frequency is limited by various physiological and developmental problems. Therefore, further enhancement in the regeneration frequency would be an added advantage in improving the *in vitro* mass propagation protocol.

It is known that ethylene, a gaseous plant growth regulator, which is involved in the regulation of various plant physiological processes, is produced by tissue in culture and can affect callus growth, shoot regeneration and somatic embryogenesis *in vitro*. (Roustan *et al*., 1990; Biddington, 1992; Pua and Chi, 1993). Polyamines (e.g., putrescine) are low-molecular weight aliphatic cation molecules prevalent in all living organisms. Although polyamines are not considered as plant hormones, they play a major role in cell division and differentiation, and have distinct physiological and developmental effect on plants (Shankar *et al*, 2011). Putrescine has been reported to promote shoot regeneration frequency in number of plant species by inhibiting the negative effect of ethylene when exogenously supplemented to the culture media as ethylene modulator (Park *et al*., 2012; Radhakrishnan *et al*., 2014). Thus in this paper, we report the influence of putrescine as regeneration enhancer in combination with cytokinin and auxin with an aim to develop a simple and high frequency regeneration protocol in *N. arbor-tristis* (L.).

MATERIALS AND METHODS

Plant Material

Surface sterilization and explant preparation

Shoot-tips were excised from fresh sprouts of two year old healthy plant of *N. arbor-tristis* maintained in Centre for Plant Biotechnology, CCS HAU New Campus, Hisar (Haryana). The explants were thoroughly washed under running tap water along with few drops of mild detergent (Teepol) for 10 min. After proper washing, shoot-tips were surface disinfected with 0.1% (w/v) $HgCl_2$ (Hi-Media, Mumbai, India) for 2 min, then rinsed five times with sterile double distilled water to remove traces of $HgCl_2$.

Culture establishment and multiplication

Surface sterilized shoot-tip explants were used to establish aseptic shoot culture on MS (Murashige and Skoog, 1962) basal media supplemented with various concentrations of plant growth regulators, 3 % (w/v) sucrose and 0.6 % (w/v) agar (Hi-Media, Mumbai, India). The pH of the medium was adjusted to 5.8 prior to autoclaving at 121°C for 20 min at 1.06 Kg cm^{-2} pressure. Putrescine (Sigma, USA) was incorporated into the autoclaved medium only after filter-sterilization using 0.22 µM filters (Sartorius Ltd., Germany).The cultures were maintained at 25 ±2°C under 16/8 h (light/dark) photoperiod with a photosynthetic light at a flux rate of 50 μEM^{-2} sec^{-1} and 50-60% relative humidity. Optimum concentration of cytokinins was standardized for efficient shoot induction by testing different concentrations (0.3-3.0 mgl^{-1}) of BAP, kinetin and zeatin (Table-1). Shoot initials obtained from optimized shoot induction medium, were subculture onto shoot multiplication media containing MS basal salts fortified with 1.0 mgl^{-1} BAP along with 0.5-3.0 mgl^{-1} GA_3 and 0.1-1.0 mgl^{-1} NAA (Table 2). For further improvement of shoot regeneration, different concentrations of putrescine (10-100 mgl^{-1}) were tested in combination with regeneration medium selected above (Table 3). After six weeks on proliferation media, the regenerated multiple shoots clumps were subcultured on to shoot elongation media (MS basal medium + 0.5 mgl^{-1} GA_3) for additional two weeks duration for elongation of shoots. Observations for percent shoot regeneration, number of shoots per explants responding and the length of shoots, were recorded after 6 and 8 weeks respectively.

Rooting and acclimatization of regenerated shoots

Elongated microshoots (>4 cm) were surgically separated and individually transferred onto liquid rooting medium for *in vitro* root induction as per the method described by Rohilla *et al*., (2015). After 4 weeks, rooted plantlets were thoroughly washed under running tap water and planted in small pots containing a mixture of soil, sand and vermicompost (1:1:1) and covered with polythene hoods to maintain about 80% relative humidity. After acclimatization in green house for two months, the hardened plants were finally transferred to natural soil under field conditions.

Scoring of data and statistical analysis

All the experiments were performed in a completely randomized design and repeated thrice with a minimum of 10 replications per treatment and data presented belong to one representative experiment. All the cultures were examined periodically and the data was recorded on the basis of visual observations. For statistical analysis the scored data were subjected to analysis of variance to detect significance of difference between means. Means differing significantly were compared using Duncan's multiple range test at P=0.05.

RESULTS AND DISCUSSION

Effect of growth regulators on shoot regeneration

An improved and effective method has been developed for the *in vitro* plant regeneration of *N. arbor-tristis*using shoot-tip explants. For establishing a plant regeneration protocol, we study the effect of different concentration of cytokinins for efficient shoot induction in *N. arbor-tristis.* Shoot initiation from shoot-tip explants did not occurs in the absence of exogenous cytokinins. Amongst different concentrations of BAP, Kinetin and Zeatin tested, BAP at 1.0 mgl^{-1} was most effective for initial shoot bud induction (Fig. 2a). Shoot buds were visible as small, nodule like protrusion from the explants after 2nd week of culture. About 76.67% explants responded, resulting in the highest (5.9 ±0.11) number of shoots per explant followed by kinetin (0.5 mgl^{-1}) that induced 5.6 ±0.12 shoots per explantwith similar *i.e.* 76.67% explant response after 30 days of inoculation (Table-1). BAP has been shown to have a similar response on shoot induction and shoot regeneration in previous studies on *Sinningia speciosa* (Park *et al*., 2012) and *Nyctanthes arbor-tristis* (Sahu *et al*., 2012). Duhan *et al*. (2014) observed maximum (100%) regeneration, when shoot tip explants were cultured on MS media supplemented with 1.5 mgl^{-1} BAP in *Lawsonia inermis.* When combination of cytokinin (1.0 mgl^{-1} BAP) with auxin (0.1-1.0 mgl^{-1} NAA) or gibberellin (0.5-3.0 $mgl^{-1}GA_3$) in MS basal media were tried for shoot proliferation study in *N.*

arbor-tristis, it was observed that all the combined treatments showed a synergistic response and the combination 1.0 mgl^{-1} BAP with 0.3 mgl^{-1} NAA in MS medium, performed best as shoot multiplication medium with highest (10.33 ±0.09) number of shoots per explant with a mean shoot length of 3.81±0.09 cm after six weeks (Fig. 1) (Table 2). The shoot number with this treatment was almost double to that with BAP alone. The results obtained are in agreement with that of Purohit *et al*, (2015), who reported maximum (2.33) number of shoots with 3.20 cm average shoot length on a medium supplemented with 1.5µM BAP, 0.5 µM NAA and 0.1µM GA_3 in *Valeriana jatamansi.* Park *et al*. (2012) also reported the synergistic response of BAP (2.0 mgl^{-1}) and NAA(0.1 mgl^{-1}) in MS medium that resulted in the highest efficiency in shoot regeneration per explant (12 ±0.8) and maximum shoot growth (1.2 ±0.1 cm) after 6 weeks in *Sinningia speciosa.*

Effect of putrescine on multiple shoot induction and shoot proliferation

Differences in regeneration frequencies among different explants are due to difference in their physiological state, endogenous level of growth regulators and their response towards growth regulators (Radhakrishnan *et al*., 2014). For improving the shoot regeneration, different concentrations of putrescine (10-100 mgl^{-1}) were tested in combination with the optimized shoot regeneration media. Exogenous inclusion of putrescine significantly promoted the morphogenetic response of shoot-tip explants of *N. arbor-tristis*. At a concentration of 50 mgl^{-1}, putrescine had maximum promoting influence on percentage of culture response (93.33%) and number of shoots (12.67 ±0.13) regenerated per explants (Fig. 2b). The percentage of culture response and number of shoots per explant increased with increasing concentrations of putrescine

Table 1. Effect of various cytokinins on shoot regeneration from shoot-tip explants of *Nyctanthes arbor-tristis* (after 30 days of Inoculation)

Plant Growth Regulators (mgl^{-1})			% Explant Responding	No. of Shoot Initials Per Explant (Mean±SE)
BAP	Kinetin	Zeatin		
Control	Control	Control	0	0.00 ±0.00 [a]
0.3			70.00	4.35 ±0.11 [ef]
0.5			76.67	5.30 ±0.18 [gh]
1.0			76.67	5.90 ±0.11 [i]
1.5			73.33	5.10 ±0.11 [g]
2.0			70.00	4.43 ±0.10 [ef]
3.0			60.00	3.22 ±0.16 [c]
	0.3		66.67	4.24 ±0.11 [e]
	0.5		76.67	5.60 ±0.12 [hi]
	1.0		76.67	5.28 ±0.17 [gh]
	1.5		70.00	4.48 ±0.13 [f]
	2.0		63.33	3.53 ±0.11 [cd]
	3.0		60.00	2.31 ±0.08 [b]
		0.3	40.00	3.62 ±0.10 [d]
		0.5	56.67	5.30 ±0.17 [gh]
		1.0	63.33	5.10 ±0.18 [g]
		1.5	60.00	4.90 ±0.12 [g]
		2.0	46.67	3.25 ±0.14 [cd]
		3.0	43.33	2.34 ±0.08 [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.

Table 2. Effect of various concentrations of different growth regulators in combination with optimal concentration of BAP (1.0 mgl^{-1}) on shoot proliferation from shoot-tipexplants of *Nyctanthesarbor-tristis* (After 42 days of culture)

Plant Growth Regulators (mgl^{-1})			% Culture	Mean No. of	Mean Shoot
BAP	GA_3	NAA	response	Shoots /Explant	Length (cm)
1.0	control	control	76.67	5.90 ±0.11 [a]	2.85 ±0.06 [b]
1.0	0.5		76.67	9.03 ±0.18 [c]	3.82 ±0.05 [c]
1.0	1.0		80.00	9.83 ±0.19 [d]	4.18 ±0.17 [d]
1.0	2.0		73.33	7.57 ±0.22 [b]	3.77 ±0.08 [c]
1.0	3.0		66.67	6.53 ±0.12 [a]	3.74 ±0.17 [c]
1.0		0.1	83.33	9.27 ±0.23 [c]	2.68 ±0.05 [b]
1.0		0.3	86.67	10.33 ±0.09 [d]	3.81 ±0.09 [c]
1.0		0.5	73.33	7.43 ±0.17 [b]	2.18 ±0.07 [a]
1.0		1.0	73.33	6.77 ±0.12 [a]	2.00 ±0.09 [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.

Table 3. Effect of ethylene modulator putrescine supplemented to multiplication media (MS basal salts + 1.0 mgl-1 BAP + 0.3 mgl-1 NAA), on shoot proliferation from shoot-tip explantsof *Nyctanthes arbor-tristis* (after 6 weeks followed by 2 additional weeks on elongation medium)

Putrescine (mgl^{-1})	% culture response	Mean No. of Shoots /Explant	Mean Shoot Length (cm)
0	83.33	10.37 ±0.21 [b]	3.98 ±0.07 [a]
10	86.67	10.67 ±0.16 [b]	6.15 ±0.08 [bc]
30	86.70	11.70 ±0.14 [c]	6.80 ±0.12 [d]
50	93.33	12.67 ±0.13 [d]	6.84 ±0.13 [d]
70	76.67	10.03 ±0.14 [a]	6.18 ±0.13 [bc]
100	73.33	9.90 ±0.14 [a]	5.94 ±0.12 [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.

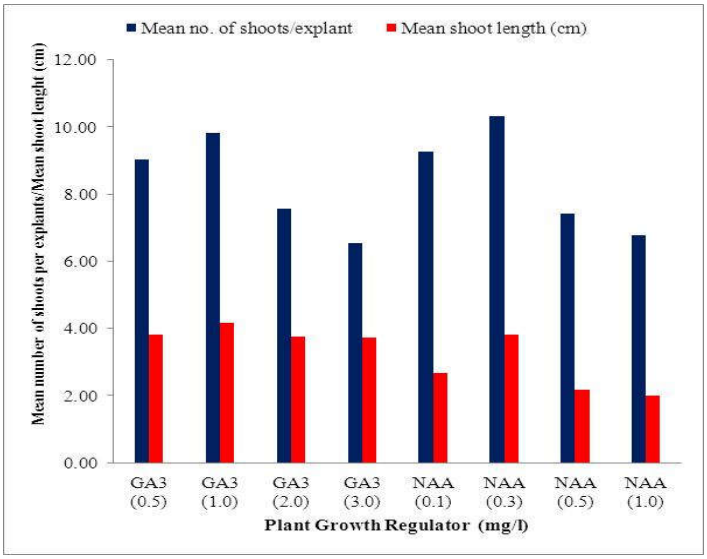

Figure 1. The evaluation of morphogenetic potential of shoot culture obtained from shoot tip explants of *N. arbor-tristis* cultured on MS basal medium supplemented with 1.0 mg/l BAP in combination with various concentrations of different plant growth regulators after 42 days

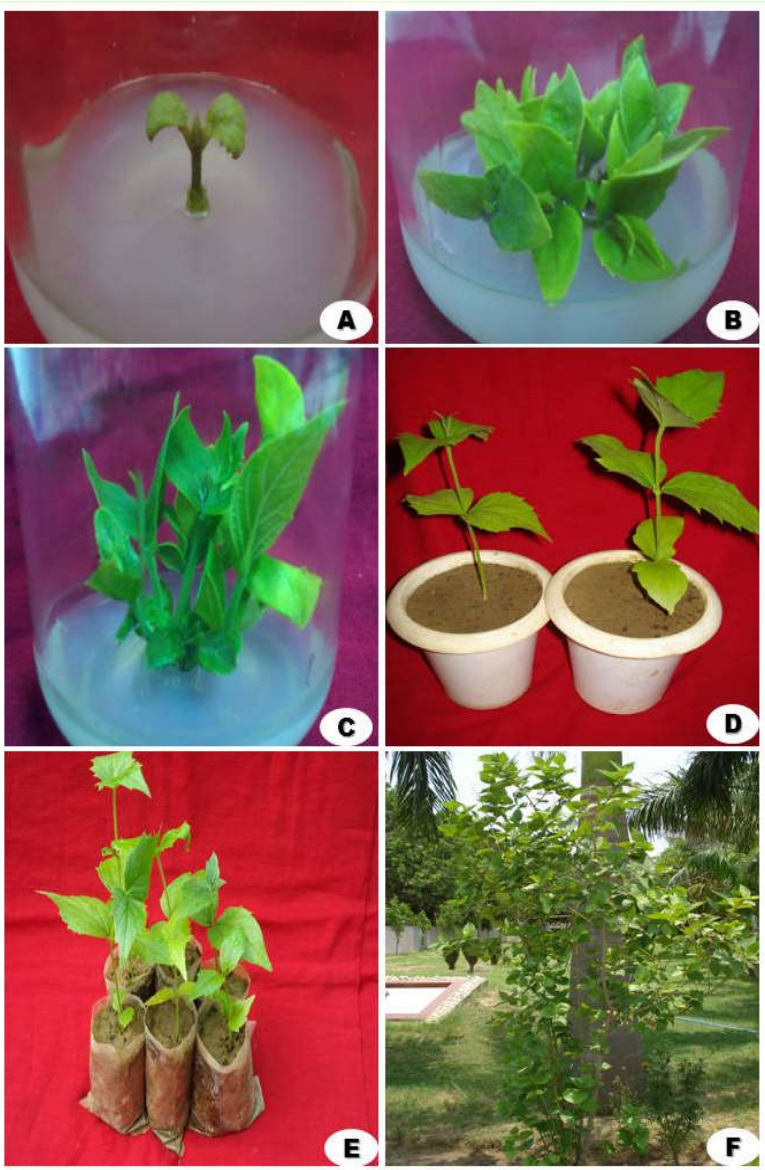

Figure 2 (A-F): *In vitro* establishment and direct regeneration in *Nyctanthes arbor-tristis* (L.) using shoot-tip explants. (A) Shoot-tip explant cultured on MS medium supplemented with 1.0 mgl^{-1} BAP after one week. (B) Shoot multiplication on MS medium supplemented with 1.0 mgl^{-1} BAP + 0.3 mgl^{-1} NAA and 50 mgl^{-1} putrescineafter 6 weeks of culture. (C) Elongation of regenerated multiple shoots on MS basal medium + 0.5 mgl^{-1} GA_3 for additional two weeks. (D) *In vitro* rooted plantlets transplanted in the thermocups containing mixture of soil, sand and vermicompost (1:1:1:). (E) Acclimatized plantlets in polybags containing natural soil. (F) Hardened plants of *Nyctanthes arbor-tristis* (L.) established under field conditions

up to an optimum concentration (50 mgl^{-1}), but there after decreased as the concentration increased (Table-3). This might be in accordance with the assumption that, when the endogenous pools of polyamines (e.g. putrescine) are at minimal levels, they may probably lead to the formation of methionine that in turn produces ethylene, and at optimum levels of putrescine (50 mgl^{-1}), they may induce organogenetic response. On the other hand, at higher level, they may divert to other biosynthetic pathways like ethylene, or feedback inhibition may even occur (Parimalan *et al.*, 2010). Multiple shoot clumps obtained from multiplication media were further subcultured onto shoot elongation media containing 0.5 mgl^{-1} GA_3, resulting in rapid elongation of shoots (Fig 2c). Highest shoot length (6.84 ±0.13 cm) was obtained from medium containing 50 mgl^{-1} putrescinein combination with other PGRs (1.0mgl^{-1} BAP + 0.3 mgl^{-1} NAA). Putrescine has already been used in other plant species for enhancing shoot regeneration frequency (Shankar *et al*., 2011; Thiruvengadam *et al*., 2012; Park *et al*., 2012; Radhakrishnan *et al*., 2014). Well elongated microshoots obtained were subjected to in vitro root induction resulting in up to 86.66% rooting. After hardening and acclimatization for six weeks, the rooted plantlets of *N. arbor-tristis* were successfully transplanted in natural soil with almost 92% field survival rate (Fig 2d-f).

Conclusion

Improving the *in vitro* shoot morphogenesis in Nyctanthes plant under the influence of an effective ethylene modulator will have an advantage. In this study putrescine has been found to enhance the shoot regeneration frequency by efficiently modulating the ethylene. Hence, putrescine can be used as regular culture media additive to increase shoot regeneration in *N. arbor-tristis.*

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