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Preconditioning of Axillary Buds in Thidiazuron-Supplemented Liquid Media Improves In Vitro Shoot Multiplication in *Nyctanthes arbor-tristis* L.

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Abstract An efficient tissue culture technology has been designed for mass multiplication of *Nyctanthes arbor-tristis* L. by preculturing nodal explants in thidiazuron (TDZ)-supplemented liquid Murashige and Skoog (MS) media. Direct inoculation of nodal segments on semi-solid MS medium augmented with various concentrations of TDZ (0.1 to 0.9 μM) produced shoots but with low regeneration response and few shoots per explant. Hence, nodal explants were pretreated with greater concentrations of TDZ (5 to 100 μM) in liquid MS media for different durations (4, 8, 12, and 16 days) with the aim of improving shoot regeneration response from cultured explants. After pretreatment, explants were transferred to agar-solidified hormone-free MS medium. Best response in terms of percent regeneration (94%), number of shoots per explant (20.00±1.15), and greatest shoot length (7.23±0.83 cm) were obtained with nodal segments pretreated in75 μM TDZ for 8 days. Similarly, root induction was obtained from pulse-treated microshoots for 24 h with 200 μM indole-3-butyric acid (IBA) followed by their transfer to 1/2 MS medium which produced an average of 5.50±0.92 roots per microshoot. The rooted plantlets were transplanted to soil with 80% success rate.

Keywords Medicinal plant · Harsinghar · Nodal explants · Pretreatment

Abbreviations

IBA	Indole-3-butyric acid
MS	Murashige and Skoog medium
TDZ	Thidiazuron

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Introduction

Nyctanthes arbor-tristis L. (Oleaceae), commonly known as harsinghar, is a valuable medicinal and ornamental plant with a potential source of an anticancerous compound—the iridoid arbortristoside A [1]. The leaf extract is used to treat arthritis, lung injury, and some painful conditions such as cancer, chronic fever, sciatica, and rheumatism [2]. Recent reports mention its hepatoprotective [3], antioxidant [4], antibacterial [5], and antidiabetic [6] properties.

It is cultivated in gardens as an ornamental tree for its fragrant white flowers with bright orange corolla tube. An extract, nyctanthin, isolated from the corolla tubes has been used as a dye. Apart from these properties, some esthetic values are also associated with this plant. The flowers are esteemed as votive offerings in the temples and made into garlands. Conventional propagation is hampered by low seed viability, poor seed germination, and scanty and delayed rooting of seedlings resulting in poor stand under natural conditions [7]. Additional pressure on the existing population has been made by pharmaceutical companies for the purpose of extraction of medicinally important metabolites, as the plants are natural reservoir of potentially useful chemical compounds which serves as drugs. Clonal collections for medicinal species are advantageous as they can facilitate the conservation of targeted lines for the pharmacological properties of the compound of interest in large quantities from representative samples. In vitro culture presents itself as an attractive tool for mass multiplication of medicinally important plant species and for the production of secondary metabolites.

There are few reports on regeneration of harsinghar employing tissue culture techniques [1, 8–10]. However, none of these protocols examines the effect of pretreatment of mature explants with growth regulators. In the present communication, a new approach of pretreatment of nodal explants with thidiazuron (TDZ) for enhanced axillary shoot proliferation has been attempted. TDZ (*N*-phenyl-*N*′-1,2,3-thidiazol-5-yl-urea) is a non-purine cytokinin compound that has been established as a potent growth regulator of morphogenetic responses in many plant species [11–14]. The present study will examine the efficiency of nodal buds in forming multiple shoots either with direct application of TDZ in Murashige and Skoog (MS) medium or preconditioning with TDZ prior to transfer to hormone-free MS medium

Materials and Methods

Plant Material

Young shoots of *N. arbor-tristis* L. collected from a 2-year-old plant maintained at the botanical garden of the university were first treated with 0.1% (*w*/*v*) Bavistin, and then washed under running tap water for about half an hour. Second step was their treatment with a liquid detergent Labolene 5% (*v*/*v*) (Qualigens, India) for 15 min followed by five to six washes with sterilized double-distilled water. The shoots were then surface sterilized with 0.1% (*w*/*v*) $HgCl_2$ solution for about 5 min followed by five to six repeated washes with sterilized double-distilled water in order to remove traces of sterilant. Nodal segments (0.5–1.0 cm) were excised aseptically and cultured in specified MS [15] medium (20 ml) in culture tubes (125×25 mm) (Borosil, India).

Culture Media and Culture Conditions

The culture media tested consisted of MS salts and vitamins with 3% sucrose and 0.8% agar or 0.25% gelrite (Qualigens, India). The pH of the media was adjusted to 5.8 with 1 N

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 of 50 μmol m^{-2} s^{-1} provided by cool white fluorescent lamps (Philips, India) with 60±5% relative humidity.

Experimental Set-up

In the first set of experiment, nodal explants were directly inoculated (without pretreatment) in semi-solid MS media supplemented with TDZ at various concentrations (0.1, 0.3, 0.5, 0.7, and 0.9 μM). After 4 weeks of incubation, cultures were transferred to fresh MS medium (hormone-free) after every 3 weeks. Shoot induction data was recorded after 4 weeks of culture while multiplication data was recorded after every subculture passages.

In second set of experiment, sterilized nodal explants were first precultured in liquid MS medium augmented with varying concentrations of TDZ (5, 25, 50, 75, and 100 μM) for different durations (4, 8, 12, and 16 days). These nodal segments were precultured in 50 ml liquid media on a rotatory shaker at 100 rpm for different time periods. MS medium lacking TDZ served as control. Following the initial pretreatment, the explants from each treatment were transferred to simple semi-solid MS basal medium after every 4, 8, 12, and 16 days for shoot induction, in order to determine the optimal exposure duration. All cultures were subcultured to fresh MS medium after every 3 weeks. The percentage of explants forming shoots, the number of shoots differentiated, and the shoot length were recorded after 8 weeks of culture.

Rooting of Shoots In Vitro

For in vitro root induction, isolated microshoots of about 4–5 cm in length with two to four fully expanded leaves were pulse-treated with different concentrations of indole-3-butyric acid (IBA; 100, 150, 200, 250, and 300 μM) for 24 h on a filter paper bridge and subsequently transferred to 1/2 MS medium without IBA, gelled with 0.25% (*w*/*v*) gelrite. Data on rooting frequency, root numbers, and root length were recorded after 4 weeks.

Acclimatization of Regenerated Plants

Rooted plantlets were removed carefully from root induction medium and washed gently with sterilized double-distilled water to remove any adherent gelrite to it. The plantlets were

Table 1 Effect of TDZ on shoot regeneration from nodal explants of *N. arbor-tristis* in MS medium after 4 weeks of culture

TDZ (μM)	% Regeneration	Number of shoots/explant	Shoot length (cm)
0.0	00	0.00±0.00	0.00±0.00
0.1	60	5.46±0.76[a]	1.83±0.29
0.3	77	6.30±0.85[a]	2.33±0.56
0.5	80	8.53±0.29[a]	3.16±0.44[a]
0.7	70	7.13±0.81	2.20±0.66
0.9	53	3.36±0.37[a]	1.03±0.12

Values represent means±SE

[a] Means that are significantly different at the 0.05 probability level using Duncan's multiple range test

Fig. 1 Direct plant regeneration from nodal buds of *N. arbor-tristis*. **a**, **b** Shoot initiation and multiplication from nodal explants on MS medium supplemented with 0.5 μM TDZ after 4 and 8 weeks of culture. **c**, **d** Shoot induction and proliferation on hormone-free MS medium from preconditioned axillary buds with 75 μM TDZ for 8 days after 4 and 8 weeks of culture. **e** A rooted plantlet in half-strength MS medium after pulse treatment with 200 μM IBA for 24 h. **f** An acclimatized plantlet in soilrite

transferred to thermocups containing sterile soilrite (Keltech Pvt. Ltd., Bangalore, India). The cups were covered with transparent polythene bags (27×18 mm) to ensure high humidity. These pots were watered with 1/2 MS salt solution after every 3 days. Polythene bags were opened after 2 weeks in order to acclimatize plants to field conditions.

Statistical Analysis

All experiments were set up in a completely randomized design and repeated thrice with a minimum of ten replicates employed for each treatment. The data were subjected to analysis of variance to detect significant difference between means. Means differing significantly

were compared using Duncan's multiple range test at $P=0.05$. All the statistical analyses were done by using SPSS version 11 (SPSS Inc., Chicago, IL, USA) statistical software package.

Result and Discussion

Shoot Induction and Multiplication

The method of multiple shoot induction using nodal explants of a mature plant was quick and resulted in a high frequency of shoot cultures. Direct shoot multiplication is preferred than organogenesis from callus as it generates true-to-type plants. In first set of experiment, the efficiency of shoot induction from nodal explant without pretreatment was analyzed on MS medium gelled with agar, augmented with lower concentrations of TDZ. Nodal explants cultured on MS medium without growth regulators did not show any morphogenetic response even after 4 weeks of culture. However, MS medium supplemented with various concentrations of growth regulators showed a major and distinctive role in shoot buds differentiation. One of the main functions of cytokinin is the release of axillary bud by the phenomenon of apical dominance, thus initiating shoot proliferation. Among all the concentrations of TDZ tried, 0.5 μM TDZ showed better shoot regeneration frequency (80%) with 8.53±0.29 shoots per explant after 4 weeks of culture (Table 1) (Fig. 1a, b). There is a linear correlation between an increase in TDZ concentration to an optimal level (0.5 μM) and shoot production. The stimulating effect of TDZ in multiple

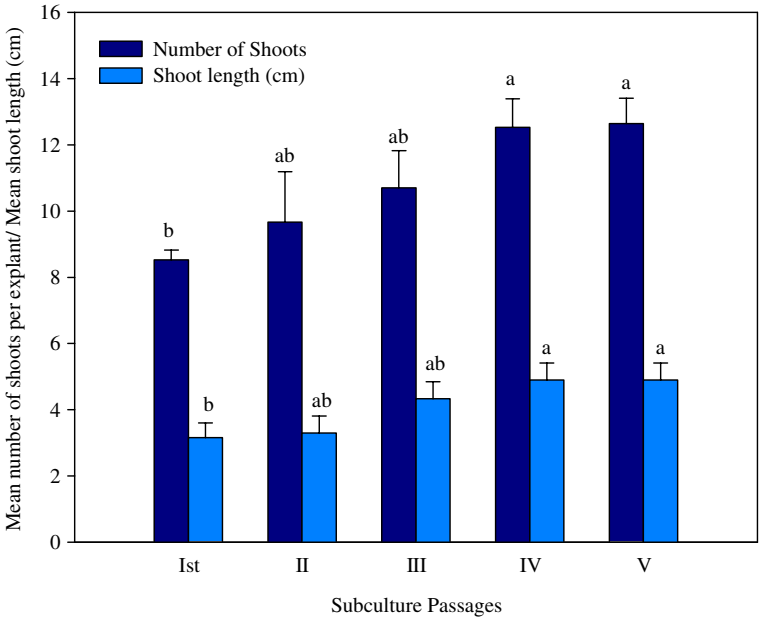

Fig. 2 The evaluation of morphogenetic potential of shoot culture obtained from TDZ (0.5 μM) after being tested for five subculture passages on a growth regulator-free MS medium. The *bars* represent mean±SE. *Bars* denoted by the *same letter* within response variables are not significantly different ($P=0.05$) using Duncan's multiple range test

Table 2 Effect of different concentrations of TDZ and duration of culture on TDZ-supplemented liquid MS medium on regeneration of nodal explants, 8 weeks after transfer to MS basal medium

TDZ (μM)	Days	% Regeneration	Number of shoots/explant	Shoot length (cm)
5	4	57	3.00±0.57	2.93±0.26
	8	63	4.06±0.69	4.60±1.84
	12	60	2.06±0.17[a]	2.06±0.17
	16	40	2.10±0.58[a]	1.46±0.26
25	4	76	3.83±0.66	3.63±0.92
	8	80	9.30±1.25	4.23±0.53
	12	60	6.40±0.80	1.56±0.72
	16	53	3.33±0.81	1.00±0.36[a]
50	4	73	6.96±0.92	4.33±0.27
	8	87	12.53±1.36	6.56±1.44
	12	80	10.33±0.90	2.50±0.40
	16	60	4.13±0.81	2.70±0.90
75	4	87	12.60±0.87	7.06±0.52
	8	94	20.00±1.15[a]	7.23±0.83[a]
	12	90	15.23±1.53[a]	5.90±1.09
	16	78	10.50±0.98	5.16±2.00
100	4	60	4.26±0.37	1.26±0.40[a]
	8	80	7.93±1.15	3.16±0.68
	12	70	5.30±0.76	1.40±0.34
	16	50	3.10±0.66	0.93±0.06[a]

Values represent means±SE

[a] Means that are significantly different at the 0.05 probability level using Duncan's multiple range test

shoot formation has been documented in a number of plant species [11, 12, 16, 17]. At higher concentrations of TDZ, a decrease in caulogenic frequency of explants with hyperhydric and distorted shoots was noticed. Our results are in accordance with the findings of Huetteman and Preece (1993) [11] where lower concentrations of TDZ were more favorable for axillary shoot multiplication while higher concentrations lead to callus formation. The numbers of shoots produced were inadequate for large-scale propagation, even subculturing led to an increase in shoot numbers but not up to an appreciable level. Therefore, a new strategy of pretreating explants with TDZ was tried to improve shoot parameters observed with experiment 1.

Although multiple shoots were induced on TDZ-supplemented media, they failed to elongate on transfer to same medium resulting in tight bud clusters with some leaf expansion. Inhibition of shoot elongation may be due to high cytokinin activity of TDZ, whereas the presence of a phenyl group may be a possible cause of shoot bud fasciation. Similar pattern of deformities have been reported in several plants including *Adhathoda beddomei* [18], *Ocimum americanum* [19], and *Rauvolfia tetraphylla* [12]. So, in order to overcome such problem, these shoot clusters were transferred to a secondary medium lacking TDZ. This type of culture procedure using primary (shoot induction) and secondary (shoot elongation) media has been successfully applied to a number of plant species, viz., *Morus alba* [20] and *Cassia angustifolia* [21]. The shoots obtained from semi-solid MS medium or from pretreated cultures with TDZ were subcultured onto hormone-free MS

Table 3 Effect of pulse treatment of IBA on in vitro root induction followed by their transfer to hormone-free 1/2 MS medium after 4 weeks of culture

IBA (μM)	% Regeneration	Number of roots/shoot	Root length (cm)
100	20	1.00±0.00[a]	1.03±0.14[a]
150	50	3.36±0.53[a]	2.30±0.36
200	80	5.50±0.92[a]	3.80±0.63[a]
250	60	4.50±0.36	2.83±0.37
300	40	1.33±0.33[a]	1.80±0.28

Values represent means±SE

[a] Means that are significantly different at the 0.05 probability level using Duncan's multiple range test

medium about five times for further multiplication and proliferation at an interval of 3 weeks. The greatest number of shoots (12.53±0.86) and the longest shoot length (4.90± 0.51 cm) were obtained from non-precultured nodal segment at fourth subculture (Fig. 2). Similar effects of subculturing were reported earlier on *Cassia angustifolia* [21] and *Cardiospermum halicacabum* [17].

In the second set of experiment, nodal explants were preconditioned with different doses of TDZ (5, 25, 50, 75, and 100 μM) in order to obtain a better multiplication rate. Pretreatment with various concentrations of TDZ in liquid medium for different durations (4, 8, 12, and 16 days) followed by their transfer to MS gelled medium without TDZ had a very significant effect on shoot induction and multiplication (Table 2). The best response was obtained at 75 μM TDZ when preculturing was done for 8 days. An average of 94% cultures responded with 20.00±1.15 numbers of shoots per explant at 75 μM TDZ followed by transfer to MS medium free from growth regulators after 8 weeks of culture (Fig. 1c, d). The technique of pretreating the explants with different plant growth regulators was employed with a view to get maximum results, as it helps in better uptake of nutrition where the explants itself is submerged in a sea of nutrients and is in direct touch with hormone [22–24]. Recently, it was reported that pretreatment of explants in TDZ was more effective than any other growth hormone and our results are in accordance with the reports on rose [25], *Curcuma longa* [26], *Curculigo orchioides* [27], and *Ocimum basilicum* [16].

Usually, lower ranges of TDZ (1 nM to 10 μM) are recommended by Huetteman and Preece [11] for shoot induction, as the shoots might be susceptible to fasciation and hyperhydricity. TDZ promotes the synthesis and accumulation of purine and also alters cytokinin metabolism to increase the levels of endogenous cytokinin by inhibiting the action of cytokinin oxidase [28, 29]. TDZ may be needed as a trigger for initiating the proliferation of shoot meristems. For quick pretreatment, higher concentrations of TDZ has been employed, i.e., up to 200 μM [25]. The influence of cytokinin pretreatment on adventitious bud formation in *Solanum melongena* [30], *Pseuditsuga menziesii* [31], and on *Musa* spp. [32] was reported. Our results are in corroboration with the studies made by Murch et al. [33] in *Hypericum perforatum*, Pranthanturaug et al. [26] in *Curcuma longa*, Mroginiski et al. [34] in *Arachis correntina*, and Siddique and Anis [16] in *O. basilicum*.

Rooting In Vitro and Acclimatization of Plantlets

In vitro rooting was found difficult for *N. arbor-tristis* micropropagation. None of the shoots rooted on full-strength MS or in its various reductions (1/2 MS, 1/3 MS, and 1/4 MS) with or without various auxin treatments (IBA, IAA, or NAA). So, a different strategy

for rooting was applied where isolated microshoots were pulse-treated with different doses of IBA for 24 h. This was followed by their transfer onto 1/2 strength MS medium lacking IBA. We obtained 80% rooting success with 200 μM IBA forming a maximum of 5.50± 0.92 roots with 3.80±0.63-cm root length after 4 weeks of culture (Table 3) (Fig. 1e). Such technique has also been reported in case of *Pterocarpus marsupium* [14], *Hagenia abyssinica* [35], and in *Cassia angustifolia* [21].

Rooted plantlets were removed from the culture vessels with minimal damage to the roots and washed under running tap water then finally planted in sterile soilrite. Survival of plantlets in the green house conditions was 80% (Fig. 1f).

Conclusion

The protocol developed in the present study proves an impressive efficiency of pretreatment of explants in liquid medium on their regenerative convertibility into shoots. The results revealed that pretreated explants are more vigorous in parameters evaluated with the untreated ones. Therefore, this technique will be useful in propagating plants on commercial scale and for genetic transformation studies.

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