In Vitro Regeneration of Coral Tree from Three Different Explants Using Thidiazuron

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ADDITIONAL INDEX WORDS. cytokinin substitute, ISSR, plant growth regulators, urea derivative

SUMMARY. The coral tree (*Erythrina variegata*) is a multipurpose horticultural plant with a plethora of medicinally important alkaloids. Regeneration via tissue culture can provide an efficient alternative to seed-grown plantlets and reduce the cost of the plant significantly. Thidiazuron (TDZ) is an efficient plant growth regulator and is effective in numerous species. However, the response to it varies with the type and position of the tissue on the plantlet treated. This study was carried out to ascertain the best tissue types for micropropagation of the coral tree using TDZ. Three tissue types (shoot tip, nodal, and hypocotyl), originating from different strata of the plantlet were evaluated. Adventitious shoots were observed in all three explants at the tested concentrations. However the quality and the shoot number varied significantly with the type of explant. Explants with a meristematic zone (shoot tip and nodal) were more responsive to the treatment compared with hypocotyl tissue lacking preexisting meristem. Nodal explants produced the maximum number of shoots (about eight) per explant after 4 weeks of culture, whereas shoot tips produced about only five shoots per explant at an equimolar concentration (1.5 μM). Approximately three shoots were observed in hypocotyl explants. Moreover, growth and rooting of the regenerated shoots was influenced by the origin of the explants. The molecular characterization of the regenerants using intersimple sequence repeat (ISSR) markers revealed genetic homogeneity among regenerants. An efficient micropropagation method for the coral tree is described.

The coral tree (*Erythrina variegata*) is an important multipurpose tree enriched with diverse medicinal properties (Jesupillai et al., 2008; Ratnasooriya and Dharmasiri, 1999; Sachin and Archana, 2009). Among horticulturists, it is a popular choice for landscaping despite its deciduous nature (Hanelt et al., 2001; Huxley, 1992). When the leaves alight the branches, it is adorned with beautiful, blooming inflorescence. Flowers produce copious

nectar, which attracts exotic birds, adding to the beauty of the landscape. The species is also used for phytoremediation of heavy metal- and salt-polluted land (Whistler and Elevitch, 2006). Because of the need for efficient and rapid propagation methods for vegetative propagation of the coral tree to fulfil an increasing demand for it, a plant tissue culture technique was attempted using benzyl adenine (BA) and α -naphthalene acetic acid (NAA) (Javed and Anis, 2015). However, TDZ has not been examined for its morphogenic response in this species. For several plant species, TDZ has been used as an efficient alternative for the combination of cytokinins and auxins. Another advantage is that, compared with other plant growth regulators (i.e., BA and NAA), less TDZ is required to get results (Ahmad and Anis, 2007; Faisal et al., 2005).

TDZ influences the metabolism and uptake of auxins positively in plant tissues (Hutchinson et al., 1996; Murch and Saxena, 2001; Murthy et al., 1995), and in some cases induces ethylene-like effects in tissue culture (Mundhara and Rashid, 2006). Therefore, TDZ has been used in tissue culture to induce a variety of responses [i.e., adventitious shoots (Jahan et al., 2011), roots (Radhakrishnan et al., 2009), organogenesis (Yao et al., 2016) and embryogenesis (Kahia et al., 2016)] at different concentrations in various species and tissues. However, invariably prolonged exposure of TDZ has been implicated in unwanted effects, such as stunting of shoots, basal callusing, and difficulty in rooting (Ahmad and Anis, 2007; Khan and Anis, 2012). The response of explants to TDZ varies with concentration, exposure duration, and the explant type. Thus, a much more efficient tissue culture system could be developed by optimizing the parameters affecting the TDZ response.

Our study was undertaken to analyze the response of different explants excised from the top, middle, and bottom of coral tree seedlings. Explants were exposed to different concentrations of TDZ to establish an efficient regeneration system.

Materials and methods

EXPLANT SOURCES. Coral tree seeds were obtained from the botanical garden of Aligarh Muslim University, Aligarh, India. Seeds were washed under running tap water using liquid detergent [5% v/v (Labolene; Glaxo Smith Kline, Mumbai, India)] for 15 min, followed by repeated washing with sterile distilled water to remove any adherent dust particles. Seeds were surface-sterilized with freshly prepared 0.1% (w/v) mercuric chloride for 3 min, followed by repeated washing with sterile double-distilled water, and germinated on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962).

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Units

To convert U.S. to SI, multiply by	U.S. unit	SI unit	To convert SI to U.S., multiply by
29,574	fl oz	μL	3.3814×10^{-5}
0.0929	ft ²	m ²	10.7639
2.54	inch(es)	cm	0.3937
6.8948	psi	kPa	0.1450
$(^{\circ}\text{F} - 32) \div 1.8$	$^{\circ}\text{F}$	$^{\circ}\text{C}$	$(^{\circ}\text{C} \times 1.8) + 32$

Fig. 1. Effect of thidiazuron on shoot proliferation from (A) nodal explants, (B) shoot-tip explants, and (C) hypocotyl explants of coral tree after 4 weeks of culture. Bars represent the number of shoots per explant; the lines represent the average shoot length. Bars and lines denote mean $\pm$ SE. Bars and lines accompanied by the same letter within a group are not significantly different at $P = 0.05$ using Duncan's multiple range test; 1 cm = 0.3937 inch.

Fig. 2. Different explants of coral tree after 4 weeks of incubation in 1.5 μ M thidiazuron-fortified Murashige and Skoog (MS) medium. (A) Shoot-tip explant with heavy basal callusing. (B) Adventitious shoot proliferation in nodal explants. (C) Hypocotyl explant with single long shoots. Shoot proliferation in (D) nodal and (E) hypocotyl explants after 4 weeks of transfer to MS basal medium.

After 14 d of germination, shoot tip, nodal, and hypocotyl explants were excised from the seedling and inoculated on regeneration media.

MEDIA AND CULTURE CONDITIONS.

The culture medium consisted of MS medium supplemented with 3% (w/v) sucrose and different concentrations of TDZ (0.0, 1.0, 1.5, 2.0, and 2.5 μ M), gelled using 0.8% (w/v) agar, and pH-adjusted to 5.8 using 1 M hydrochloric acid or sodium hydroxide before autoclaving at 121 $^{\circ}$ C at 15 psi for 15 min. MS medium devoid of TDZ was used as a control. All the responsive cultures were transferred to the control medium after 4 weeks of incubation on TDZ-supplemented media. Effect of exposure duration was evaluated by growing three batches (10 explants each) of culture at the optimum level of TDZ and transferring them to basal media after 4, 6, or 8 weeks. For rooting, elongated shoots were excised individually and transferred to half-strength MS medium augmented with 2.5 μ M indole-3-butyric acid (IBA) (Javed and Anis, 2015). Data were recorded every 2 weeks of incubation for percentage response and roots per shoot. All cultures were maintained at 24 ± 2 $^{\circ}$ C under a 16-h photoperiod with a photosynthetic photon flux density of 50 μ mol $\text{m}^{-2} \cdot \text{s}^{-1}$ provided by cool white fluorescent lamps (Philips India, Gurugram, India) and 65% relative humidity (RH).

ACCLIMATIZATION. Plantlets with a well-developed shoot and roots were transferred to plastic cups containing

sterile substrate (Soilrite®; Flora Bio-tech, New Delhi, India) and kept under culture room conditions. Plants were covered using plastic bags to ensure high humidity and were irrigated every other day with half-strength MS basal solution devoid of organic supplements. The polythene bags were removed gradually by piercing small holes in them over a period of 2 weeks. After 4 weeks, plantlets were transferred to garden soil (1:4, manure:soil by volume) and maintained in a greenhouse (RH, 50% $\pm$ 20%) for 4 weeks before field transfer.

MOLECULAR CHARACTERIZATION. Genomic DNA was extracted from 10

tissue culture-derived plantlets and the donor plant using the cetyl-methylammonium bromide method, as described by Doyle and Doyle (1990). For ISSR marker analysis, UBC primers (developed by University of British Columbia, Vancouver, BC, Canada) were used. Polymerase chain reactions (PCRs) were performed in a thermocycler (TGradient ThermoBlock; Biometra, Gottingen, Germany). A total of 10 $\times$ buffer (2 μ L), 2500 μ M magnesium chloride (1.2 μ L), 1000 μ M deoxyribonucleotide triphosphates (0.4 μ L), 2 μ M primer, 3 U Taq polymerase (0.2 μ L), and 50 ng template DNA was

mixed to prepare the PCR amplification mixture (20 μ L). For PCR amplification, the thermocycler was programmed for 38 cycles, each including a 94 °C denaturation step of 5 min, an annealing step at 55 °C for 1 min, and an elongation step at 72 °C for 1 min. At the end of the last cycle, a final extension was carried out at 72 °C for 10 min. DNA amplicons were electrophoresed at 50 V for 2 h in 0.8% (w/v) agarose gel, then visualized by ethidium bromide using an ultraviolet transilluminator (Bio-Rad Laboratories, Hercules, CA).

STATISTICAL ANALYSIS AND DATA COLLECTION. Experiments were carried out using a complete randomized block design and each experiment was repeated three times with 10 replicates per treatment. Data for the measured parameter were recorded every 2 weeks. The data were subjected to analysis of variance and Duncan's multiple range test ($P = 0.05$) for post hoc analysis. Analysis was done using the statistical software package SPSS (version 11; IBM Corp., Armonk, NY).

Results

EFFECT OF TDZ CONCENTRATION. TDZ induced adventitious shoot proliferation successfully in explants. However, the number of shoots produced and their length varied (Fig. 1). Invariably, the 1.5- μ M concentration evoked the best response regardless of the explant type (Fig. 1). At greater concentrations, the cultures developed heavy basal callusing, with fewer shoots and a reduced height. Rosette formation was detected frequently in explants growing

Fig. 3. Differential response varying with explant types of coral tree and concentrations of thidiazuron. Bars denote mean $\pm$ SE.

Fig. 4. Effect of different thidiazuron exposure time on nodal explant of coral tree (A) after 4 weeks, (B) after 6 weeks, and (C) after 8 weeks of exposure. Bars denote mean $\pm$ SE.

in greater concentrations; shoots developed were clustered and swelled to assume a hyperhydrated appearance (Fig. 2). However, at an equimolar TDZ concentration (1.5 μM), explants exhibited a differential response, with nodal explants producing an average of 7.7 shoots per explant in 89% of the cultures, whereas shoot-tip and hypocotyl explants produced 5.1 and 3.0 shoots per explant in 83% and 81% of cultures, respectively, after 4 weeks of incubation (Fig. 3).

EFFECT OF TDZ EXPOSURE TIME. Cultures incubated for a period of 4 weeks in the 1.5- μM TDZ concentration produced the maximum shoots per explant. Thereafter, there was a cessation of longitudinal growth in the cultures, and continued incubation (up to 8 weeks) in TDZ-supplemented media induced deformities, such as heavy basal callusing, hyperhydricity, and blackening. The TDZ was withdrawn from the culture media, and basal media were used for further growth. A TDZ exposure of 4 weeks was found to be optimal, producing a maximum of 12 shoots per explant 4 weeks after transfer. Exposure of 6 or 8 weeks produced about 10 and 7 shoots per explant (Fig. 4).

EFFECT OF EXPLANT TYPE. Maximum shoot numbers were obtained on the nodal explants (Fig. 2B). However, shoot length did not increase until cultures were transferred to a TDZ-free MS media. In the cultures emanating from hypocotyl explants, one of the shoots grew longer whereas the others remained very short. Heavy basal callusing was observed in the shoot-tip explants (Fig. 2A) and nodal explants, but it was not so conspicuous in the hypocotyl explants (Fig. 2C). However, the responsiveness of all explants was similar at the 1.5- μM concentration. A similar trend was observed even after the withdrawal of TDZ from the media (Fig. 4).

ROOTING. The regenerated shoots obtained from different sources (explant) were exposed separately to the rooting media. Shoots obtained from hypocotyl explants were more responsive to the media, whereas the shoot-tip-derived shoots were least responsive. Rooting was observed in the shoots after 4 weeks of incubation on $\frac{1}{2}$ MS medium supplemented with IBA (2.5 μM). After 4 weeks of incubation, 51% of

the shoots derived from hypocotyl explants rooted successfully, with an average of 2.9 roots per shoot (Fig. 5). However, only 42% and 38% of the shoots derived from the nodal and shoot-tip explants, respectively, could root (with 1.9 and 1.5 roots per shoot, respectively) after 4 weeks of incubation. No significant variations were observed in root length (Table 1).

MOLECULAR CHARACTERIZATION. The regenerants were assayed for genetic variability using ISSR markers. DNA of the donor and regenerated plants responded with 7 of the 10 markers tested with clear and reproducible bands. These seven ISSR primers produced 57 distinct and readable bands. The number of reproducible bands for each primer varied from 5 to 10 (Table 2). No polymorphism could be detected in the assay. Banding profiles of the regenerants were monomorphic and similar to that of donor plant.

Discussion

The effects of TDZ were evaluated on adventitious shoot induction and proliferation from different explants of coral tree. TDZ induced adventitious shoots in all the explants tested. However responses varied with TDZ concentration and explant type. The effectiveness of any particular concentration depends mostly on the species and type of tissue. Huettelman and Preece (1993) proposed that TDZ was effective in the concentration range of 0.1 to 10 μM , varying significantly among species, and our results corroborate these observations. TDZ has cytokinin-like activity, showing strong affinity to cytokinin receptors despite lacking a purine structure (Spichal et al., 2004). Hence, TDZ has been used extensively as a plant growth regulator in plant cell, tissue, and organ culture

for some of the most recalcitrant species, inducing organogenesis or shoot proliferation successfully (Murthy et al., 1998). A concentration-dependent increase in the number of shoots was recorded up to an optimum concentration (1.5 μM) of TDZ. Among the tested concentrations, 1.5 μM was found to be optimum and, hence, most effective.

Exposure duration was a significant factor influencing the response of the explants. A 4-week exposure to TDZ (1.5 μM) was most beneficial for

Fig. 5. Rooting in regenerated microshoot of coral tree on $\frac{1}{2}$ Murashige and Skoog medium supplemented with 2.5 μM indole-3-butyric acid after 4 weeks of incubation.

Table 1. Effect of 4 weeks of incubation on $\frac{1}{2}$ Murashige and Skoog's medium supplemented with 2.5 μM indole-3-butyric acid on rooting of in vitro raised microshoots originating from different explants of coral tree.

Explant	Rooting (%)	Roots [mean $\pm$ SE (no./shoot)]	Root length [mean $\pm$ SE (cm)] ^z
Hypocotyl	51	2.90 $\pm$ 0.15 a ^y	1.2 $\pm$ 0.22 b
Nodal	42	1.90 $\pm$ 0.09 b	1.5 $\pm$ 0.33 a
Shoot tip	38	1.50 $\pm$ 0.09 c	1.0 $\pm$ 0.22 c

^z1 cm = 0.3937 inch.

^yDifferent letters within a column indicate that the values are significantly different according to Duncan's multiple range test ($P < 0.05$).

Table 2. List of intersimple sequence repeat primers used for genetic variability assay, with corresponding sequences and number of reproducible bands received from 10 randomly selected samples of coral tree regenerated via tissue culture.

Sample no.	Primer	Primers sequences (5'-3') ^z	Bands (n)
1	UBC-801	(AT) ₈ T	5
2	UBC-811	(GA) ₈ C	6
3	UBC-825	(AC) ₈ T	9
4	UBC-827	(AC) ₈ G	8
5	UBC-834	(AG) ₈ YT	6
6	UBC-855	(AC) ₈ YT	8
7	UBC-868	(GAA) ₆	0
8	UBC-880	(GGGGT) ₃ G	0
9	UBC-891	HVHT (GT) ₆ G	0
10	UBC-900	ACTTCCCCACAGGTTAACAC	5

^zH = (A, G, T); V = (A, C, G); Y = (C, T).

inducing maximum shoot proliferation. Prolonged exposure produced detrimental effects along with attrition in shoot number. Similar observations have also been reported in other species, such as the devil-pepper tree (*Rauvolfia tetraphylla*) and the arabian lilac (*Vitex trifolia*) in which over- or prolonged exposure of TDZ resulted in various deformities in culture, including hyperhydricity and fasciation in shoots. These deformities in cultures might have been caused by the effect of TDZ on auxin transport (Murch and Saxena, 2001).

In our study, the type of explant tissue influenced the kind of response from the tissue to TDZ and, consequently, the success of the regeneration protocol. The nodal explants were most responsive to the TDZ treatment, whereas the shoot-tip and internode explants produced fewer shoots per explant. The requirements and the competence of the cells of different tissues vary, as does the response to a particular growth regulator. The major reasons for this differential response are the varying levels of endogenous hormones and the level of differentiation of cells (Yan et al., 2009). Nodal explants have been used successfully with TDZ for regeneration in the golden trumpet [*Allamanda cathartica* (Khanam and Anis, 2018)].

Our observations with regard to disparity in shoot length in culture originating from the hypocotyl explant, in which one shoot was much longer than the rest of the shoots, can be equated to the epinastic response in plants, and can be attributed to enhanced ethylene production. TDZ has been reported to increase endogenous

auxin, cytokinin, and ethylene in plants (Murthy et al., 1995; Yip and Yang, 1986).

Plant tissue culture conditions are stressful and may sometimes induce genetic variations in the regenerated plants, known as somaclonal variations (Larkin and Scowcroft, 1981). The chances of somaclonal variations are increased if the cultures are exposed to stress. For micropropagation, clonal fidelity of the regenerants is an important criterion (Roy et al., 2012). Therefore, it is essential to screen the regenerants for any genetic variations. Molecular characterization of the regenerants did not reveal any genetic variation caused during the micropropagation process. Therefore, the protocol is suitable for clonal propagation using TDZ without any apprehension of genetic modification during culture.

Conclusions

Our study resulted in an efficient method for using TDZ for micropropagation of the coral tree. The cost of a coral tree plantlet is around \$6 to \$60 per unit in the international market (Alibaba Group Holding, Zhejiang, China). This cost can be reduced dramatically using our protocol. The nodal segments were most suitable for adventitious shoot production using TDZ. Use of TDZ successfully circumvented the use of cytokinin and auxin for multiplication, thus reducing time and cost.

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