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High Frequency Multiple Shoot Regeneration and Plantlet Formation in *Cassia angustifolia* (Vahl.) Using Thidiazuron

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ABSTRACT

An *in vitro* propagation system for *Cassia angustifolia* Vahl. has been developed. *De novo* shoot organogenesis was induced by culturing cotyledonary node explants excised from seven days-old aseptic seedlings. Of the different concentrations (0.1,0.3, 0.5, 0.8, 1.0, 2.5, 5.0, 7.5 and 10.0 µM) of thidiazuron (TDZ) evaluated as supplements to Murashige and Skoog (MS) medium, TDZ at an optimal concentration of 1.0 µM was effective in inducing multiple shoots. The number of shoots increased significantly when the responding explants were transferred to a medium devoid of TDZ. The highest number of shoots and shoot length were recorded at the end of fourth subculture. *Ex vitro* rooting was achieved when the basal cut end of regenerated shoots were dipped in 200 µM indole-3-butyric acid (IBA) for half an hour followed by their transplantation in plastic pots filled with sterile Soilrite^{TM} where 80% plantlets grew well and all exhibited normal development.

Keywords: adventitious shoots, conservation, *ex vitro* transplantation, Fabaceae, senna, tissue culture
Abbreviations: BA, 6-benzyladenine; **IAA**, indole -3- acetic acid; **IBA**, indole-3-butyric acid; **2-iP**, 2-isopentenyl adenine; **Kin**, Kinetin; **MS**, Murashige and Skoog medium; **NAA**, α–naphthalene acetic acid; **PPFD**, photosynthetic photon flux density; **TDZ**, thidiazuron

INTRODUCTION

Cassia angustifolia commonly known as senna is a medicinally valuable drought resistant shrub of the family Fabaceae. It is a native of Saudi Arabia and has been naturalized in India. Senna is employed in the treatment of amoebic dysentery, as an anthelmintic and as a mild liver stimulant (Anonymous 1992). Senna is a powerful cathartic used in the treatment of constipation, working through a stimulation of intestinal peristalsis. The drug mainly comprises of dried leaves and pods. The active chemical components of the plant are anthraquinone glycosides "Sennosides" especially Sennosides A and B which are responsible for the purgative action. The plant has been put in the priority list of National as well as State Medicinal Plant Board for development. It is one of the principal herbal drug having export potential for developed countries.

Conventional methods of propagation of this plant are limited to seeds. However, reduced span of viability, low germination rate and high degree of genetic heterogeneity in *Cassia* restrict its propagation through seeds. Strategies to regenerate plants from tissue cultures of legumes have been evolving steadily during the past few years.

In recent years, much attention has been given to the micropropagation of medicinal plants (Rout 2002; Faisal *et al*. 2005; 2006a, 2006b) as sources of curative compounds for several ailments. Direct regeneration or regenerant differentiation via callus cultures and subsequent whole plant regeneration are essential for an efficient utilization and application in various biotechnological techniques for plant improvement. There are few reports on the plant regeneration of *C. angustifolia* via cotyledonary node and nodal explants (Agrawal and Sardar 2003, Siddique and Anis 2007), cotyledon- and leaflet-derived calli (Agrawal and Sardar 2006) with plant growth regulators such as BA, Kin, TDZ, IAA, IBA and NAA but there is no report on the effect of thidiazuron (TDZ) on plant regeneration using cotyledonary node explants. The existing protocols yielded a maximum of 12 shoots which can be further enhanced for large scale production and cultivation of its elite clones. 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.

MATERIALS AND METHODS

Establishment of aseptic seedlings

Authenticated seeds of *C. angustifolia* obtained from the Regional Research Institute of Unani Medicine (RRIUM) Aligarh, India, were washed thoroughly under running tap water for 30 min, to remove adherent particles, treated with a detergent, Teepol (5% v/v) for 20 min, followed by washing in tap water and rinsed five times with sterile double distilled water. The seeds were surface disinfected for 4 min in 0.1% (w/v) $HgCl_2$ and finally rinsed five times with double distilled water and inoculated in Murashige and Skoog (MS 1962) medium for germination. Cotyledonary node excised from seven days old aseptic seedlings were used as explants.

Culture media and conditions

The culture medium used for the present work was MS medium supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar. The medium was further supplemented with different concentrations of TDZ (Sigma Aldrich Co, USA) for shoot bud induction. The culture vials containing media were autoclaved at 121°C and 1.06 Kg cm^{-2} for 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 65% relative humidity.

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Short Communication

Multiple shoot induction and proliferation

The surface-disinfected explants (cotyledonary nodes) were placed on MS medium supplemented with different concentrations of TDZ (0.1, 0.3, 0.5, 0.8, 1.0, 2.5, 5.0, 7.5 and 10.0 µM) for multiple shoot induction. MS medium lacking growth regulators served as control. After an induction period of 4 weeks on TDZ enriched medium, responsive explants were inoculated onto the same basal medium but without growth regulator. All cultures were transferred to fresh medium after every 2 weeks. The 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 and their basal portion were dipped in different concentrations of IBA (50, 100, 150, 200, 250 and 500 µM) for half an hour and subsequently planted in plastic pots containing sterilized garden soil, Soilrite™ or vermiculite (Keltech Pvt. Ltd. Bangalore) 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, mean 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 response of cotyledonary node explants to TDZ is summarized in **Table 1**. Culture medium devoid of growth regulators failed to stimulate bud break in explants even when the cultures were maintained beyond 4 weeks of induction period. The explants in the control remained fresh and green for about 2 weeks, but thereafter started to blacken and eventually died. All concentrations of TDZ facilitated adventitious shoot bud induction and their subsequent proliferation. TDZ exhibits strong cytokinin-like activity and is generally effective at lower concentrations where it promotes the proliferation of axillary shoots, stimulates adventitious organ regeneration and induces somatic embryogenesis. The stimulating effect of TDZ on multiple shoot formation has been reported earlier for some woody species (Huetteman and Preece 1993) in which TDZ (0.1 nm - 10 µM) induced high rates of shoot multiplication. Tiwari *et al*. (2001) compared BA, Kin, TDZ and 2-iP in *Bacopa monniera* and achieved maximum number of shoots on 6.8 µM TDZ from nodal explants whereas Faisal *et al*. (2005) reported optimum shoot regeneration on MS me-

Table 1 Effect of different concentrations of TDZ on multiple shoot induction from cotyledonary node explants of *C. angustifolia* in MS medium after 4 weeks of culture.

TDZ (µM)	% Regeneration	№ of shoots /explant	Shoot length (cm)
0.1	53	1.7 ± 0.31 e	1.4 ± 0.14 d
0.3	60	2.7 ± 0.26 de	2.1 ± 0.20 cd
0.5	67	4.5 ± 0.32 c	2.7 ± 0.32 bc
0.8	75	5.7 ± 0.34 b	2.9 ± 0.23 b
1.0	84	8.1 ± 0.66 a	3.8 ± 0.32 a
2.5	78	6.3 ± 0.43 b	3.4 ± 0.26 ab
5.0	72	4.1 ± 0.37 c	2.8 ± 0.23 bc
7.5	68	3.3 ± 0.34 cd	1.9 ± 0.14 d
10.0	61	2.0 ± 0.23 e	1.3 ± 0.20 d

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 Evaluation of morphogenetic potential of shoot culture obtained from TDZ (1.0 µM) after being tested for six subculture passages on growth regulator-free MS medium.

Subculture passages	№ of shoots /explant	Shoot length (cm)
1st	8.7 ± 0.64 c	4.8 ± 0.23 c
2nd	11.5 ± 0.78 b	5.5 ± 0.29 ab
3rd	15.1 ± 1.01 a	5.9 ± 0.34 a
4th	17.6 ± 1.18 a	6.4 ± 0.37 a
5th	17.6 ± 1.18 a	6.4 ± 0.37 a
6th	12.5 ± 0.88 b	5.3 ± 0.30 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 3 Efficiency of root induction from shoots of *C. angustifolia* dipped in IBA solution for half an hour before field transfer. Data were taken after four weeks of transplantation.

IBA (µM)	% Rooting	№ of roots /shoot	Root length (cm)
50	NR	NR	NR
100	50	1.7 ± 0.29 c	1.6 ± 0.29 c
150	59	3.6 ± 0.31 a	2.9 ± 0.23 b
200	65	4.0 ± 0.43 a	3.9 ± 0.28 a
250	54	3.3 ± 0.31 ab	2.5 ± 0.20 bc
300	46	2.4 ± 0.23 bc	2.0 ± 0.23 c

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.
NR; no response

Table 4 Evaluation of different planting substrates for hardening off *in vitro* raised plantlets of *C. angustifolia*. Data were recorded after 4 weeks of transfer to planting substrates.

Planting Substrate	№ of plants transferred	№ of surviving plants	% Survival
Garden soil	60	40	66.6 ± 1.90 c
Soilrite™	60	54	90.0 ± 3.54 a
Vermiculite	60	49	81.6 ± 2.15 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.

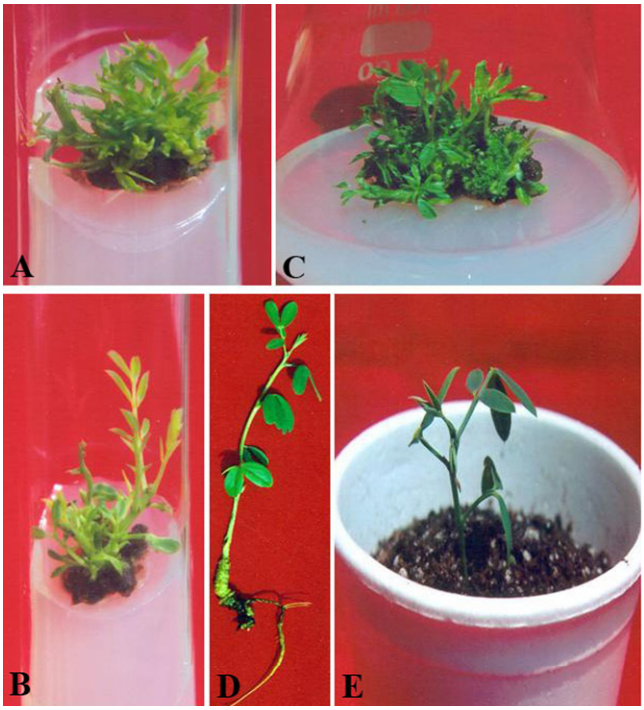

Fig. 1 ***In vitro*** **plant regeneration of** ***Cassia angustifolia*****.** (**A, B**) Multiple shoot induction on MS + TDZ (1.0 µM); (**C**) Shoot multiplication on TDZ free MS medium at fourth subculture; (**D**) *Ex vitro* rooted plantlet; (**E**) An acclimatized plant in Soilrite™.

dium supplemented with 5 µM TDZ in *Rauvolfia tetraphylla*. Siddique *et al*. (2006) studied two cytokinins, viz. TDZ and BA in *Nyctanthes arbor-tristis* and recorded maximum multiplication in 1.0 µM TDZ from cotyledonary node explants.

Of the various levels of TDZ tested, 1.0 µM proved to be most effective and optimum for inducing maximum percent regeneration (84%), maximum number of shoots (8.1 ± 0.66) and maximum shoot length (3.8 ± 0.32) (**Figs. 1A, 1B**). On lowering the concentration of TDZ from 1.0 to 0.5 µM, the number of shoots per culture was reduced. Similarly, at a higher concentration (10.0 µM), the percent regeneration as well as number of shoots was drastically reduced (**Table 1**). A reduction in the number of shoots generated from each explant at TDZ concentration higher than the optimal level was also reported in *Hypericum perforatum* (Murch *et al*. 2000), *Arachis correntina* (Mroginski *et al*. 2004) and *Cassia angustifolia* (Siddique and Anis 2007).

After an induction period of 4 weeks, the shoot multiplication rate was increased when each responsive explant was transferred to a hormone-free basal medium. However, 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 (Murthy *et al*. 1996), *Rauvolfia tetraphylla* (Faisal *et al*. 2005) and *Capsicum annuum* (Siddique and Anis 2006) has also been reported.

The highest number of shoots (17.6 ± 1.18) and shoot length (6.4 ± 0.37) were recorded up to the fourth subculture (**Fig. 1C**), which became stable during the fifth subculture (**Table 2**) and beyond which a gradual decline in multiplication rate was noticed. A similar effect of subculturing has also been reported in *Bacopa monniera* (Tiwari *et al*. 2001) and in cranberry (Debnath and McRae 2001).

The success and cost effectiveness of micropropagation relies on the rooting percentage and survival of the plantlets in field conditions. *Ex vitro* rooting was attempted as a means to decrease the micropropagation cost and also the time from laboratory to field. The best result for rooting was recorded when shoots were dipped in IBA (200 µM) as it gave the maximum frequency of rooting (65%), number of roots (4.0 ± 0.43) and root length (3.9 ± 0.28) (**Table 3**) (**Fig. 1D**). Shoots induced by TDZ and subsequently rooted *ex vitro* has also been reported in *Capsicum annuum* (Ahmad *et al*. 2006; Siddique and Anis 2006), *Nyctanthes arbor-tristis* (Siddique *et al*. 2006) and *Cyamopsis tetragonoloba* (Ahmad and Anis 2007). The rooted plantlets were successfully hardened off inside the growth room in selected planting substrate for 4 weeks and eventually established in natural soil. Among the three different types of planting substrates examined, 90% of plants survived in Soilrite™ (**Fig. 1E**) (**Table 4**) and about 80% survived following transfer from Soilrite™ to natural soil. There was no detectable variation among the potted plants with respect to morphological and growth characteristics.

Rapid and efficient multiplication rate, *ex vitro* rooting and successful transfer of plantlets to the greenhouse makes this protocol suitable for large scale multiplication as well as *ex situ* conservation of this important medicinal plant. The attempt opens the way to scale up studies to enhance biomass growth and eventually Sennoside production with cotyledonary node culture.

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