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In Vitro Adventitious Shoot Regeneration via Indirect Organogenesis from Petiole Explants of *Cassia angustifolia* Vahl.—a Potential Medicinal Plant

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Abstract An effective protocol was developed for in vitro regeneration of the *Cassia angustifolia* via indirect organogenesis from petiole explants excised from 21-day-old axenic seedlings. Organogenic callus were induced on Murashige and Skoog (MS) medium supplemented with 5.0 μM 2,4-dichlorophenoxy acetic acid and 2.5 μM thidiazuron (TDZ). Adventitious shoot regeneration was achieved on MS medium supplemented with 5.0 μM TDZ as it induced 8.5±0.98 shoots in 85% cultures. The number of shoots and shoot length was significantly enhanced when cultures were subcultured on auxin–cytokinin-containing medium. The highest number of shoots (12.5±1.10) and shoot length (4.3±0.20 cm) was recorded on MS medium supplemented with 5.0 μM TDZ and 1.5 μM indole-3-acetic acid. Regenerated shoots were rooted best on MS medium supplemented with 10.0 μM indole-3-butyric acid followed by their transfer to liquid MS filter paper bridge medium. The plants were successfully hardened off in sterile soilrite followed by their establishment in garden soil with 70% survival rate. The plants showed normal morphological characteristics similar to the field grown plants.

Keywords Adventitious · Indole-3-butyric acid · Petiole · Pulse treatment · Sennosides · Shoot bud

Introduction

Cassia angustifolia Vahl., commonly known as Senna, is a medicinally valuable drought-resistant shrub of family Fabaceae and is mainly grown as a cash crop in various parts of

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the world [1]. Senna is cultivated in Somalia, Arabian Peninsula, and near the Nile River [2]. The leaves and pods contain sennosides A, B, C, D, kampferol, anthraquinone, essential oil, isohamnentin, and emodin. Senna is employed in the treatment of amoebic dysentery as an anthelmintic and as a mild liver stimulant. Senna is a powerful cathartic used in the treatment of constipation working through a stimulation of intestinal peristalsis. 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 drugs having export potential for developing countries.

C. angustifolia is exploited heavily from wild conditions by pharmaceutical companies and local tribes throughout India for medicinal purposes. Conventionally, it is propagated by seeds. However, low germination percentage and poor viability restricts its propagation on large scale. It has therefore become imperative to develop methods for its large-scale propagation employing tools of plant biotechnology. Direct regeneration or regenerant differentiations via callus cultures and subsequent whole plant regeneration are essential for an efficient utilization and application in various biotechnological techniques for plant improvement.

Direct regeneration through cotyledonary node and nodal segment explants followed by successful ex vitro establishment has already been reported earlier [3–5]. Few reports on plant regeneration via cotyledon and leaflet derived calli [6] and somatic embryogenesis from cotyledon [7] are available, but there is no report of indirect organogenesis from petiole explants. Therefore, in the present investigation, an attempt has been made to evaluate the choice of auxin and cytokinin concentrations and their combinations in order to develop a standard reproducible protocol for rapid adventitious shoot regeneration and propagation from petiole explants of *C. angustifolia*. Regeneration via indirect organogenesis will also be useful for genetic transformation studies and production of somaclonal variants in those medicinal plants having higher secondary metabolite content.

Materials and Methods

Plant Material and Establishment of Aseptic Seedlings

Seeds from mature pods of healthy plants grown in Arid Forest Research Institute, Jodhpur, India were used for this study. The seeds were washed in running tap water followed by soaking in 5% (*v*/*v*) solution of a detergent, Labolene (Qualigens, Mumbai, India) for 5 min. Following repeated washes with sterile distilled water, the seeds were inoculated in Murashige and Skoog (MS) [8] medium for germination. Petiole explants excised from 21 days old aseptic seedlings were used as explants.

Culture Media and Culture Conditions

The culture media tested was MS with 3% sucrose (*w*/*v*) and 0.8% (*w*/*v*) agar. Plant growth regulators and their combinations were added to the medium as specified below. The pH of the MS was adjusted to 5.8 by using 1 N NaOH or 1 N HCl prior to autoclaving at 121 °C for 20 min. All the cultures were maintained in a culture room at 24±2 °C under a 16-h photoperiod with a photosynthetic photon flux density of 50 μmol $m^{-2} s^{-1}$ provided by cool white fluorescent lamps (Phillips, India) and 60–65% relative humidity.

Callus Induction

For callus induction, explants were placed on MS medium supplemented with different concentrations of 2,4-dichlorophenoxy acetic acid (2,4-D; 0.5, 2.5, 5.0, and 10.0 μM) and thidiazuron (TDZ; 0.1, 0.5, 2.5, and 5.0 μM). After 4 weeks of culture, the frequency of explants producing callus was scored. Subsequently, the induced calli were proliferated onto fresh medium with 5.0 μM 2,4-D and 2.5 μM TDZ. Callus cultures were subcultured after every 4 weeks, and data were recorded after 8 weeks of culture.

Shoot Organogenesis from Callus

Well-established green and compact callus (~0.5 g) grown for 8 weeks on MS medium supplemented with 5.0 μM 2,4-D and 2.5 μM TDZ was used for shoot organogenesis. Calluses were transferred to shoot organogenesis media consisting of MS basal medium supplemented with benzyl adenine (BA), TDZ, and kinetin (Kin; 0.5, 2.5, 5.0, 7.5, and 10.0 μM) either singly or in combination with indole-3-acetic acid (IAA) and α-naphthalene acetic acid NAA (0.5, 1.0, 1.5, 2.0, and 2.5 μM). Cultures were transferred onto fresh media after every 2 weeks. The percentage response of shoot organogenesis from callus, mean number of shoots, and mean shoot length were recorded after 8 weeks of transfer of callus to shoot organogenesis media.

Rooting of Shoots In Vitro

Elongated healthy shoots (3–4 cm) were excised and cultured on root induction media comprising MS medium supplemented with indole-3-butyric acid (IBA) at different concentrations (0.5, 2.5, 5.0, 10.0, and 12.5 μM) for 2 weeks followed by their transfer to MS liquid filter paper bridge media without IBA. Data were recorded on percentage of rooting, number, and length of roots after 4 weeks of culture.

Hardening and Acclimatization of Regenerated Plantlets

Rooted plantlets were removed from root induction medium, and the rooted shoots were washed in sterile distilled water to remove basal callus. The plantlets were then transferred to plastic pots (6 cm diameter) containing sterile soilrite (Keltech Pvt. Ltd., Bangalore, India). The plastic pots were covered with polythene bags to maintain the relative humidity. These pots were maintained at 24±2°C and with a 16-h light photoperiod 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. After 4 weeks, acclimatized plants were transferred to pots containing normal garden soil and maintained in a greenhouse under natural light.

Statistical Analysis

All the experiments were set up in a completely randomized design and repeated three times with a minimum of ten explants per treatment before conducting the statistical analysis. 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 Ver. 11 (SPSS Inc., Chicago, IL, USA) statistical software package.

Table 1 Effect of different concentrations of 2,4-D and TDZ on callus induction from petiole explants after 8 weeks of culture.

Plant growth regulators (μM)		% Response
2,4-D	TDZ	
0.5		40.8±1.92d
2.5		52.3±2.00c
5.0		60.8±3.48b
10.0		45.8±1.52d
5.0	0.1	65.0±3.02b
5.0	0.5	72.0±3.98ab
5.0	2.5	81.8±4.01a
5.0	5.0	59.7±3.01bc

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

Results and Discussion

Callus Induction

Dark green callus induction was recorded from the cut ends of petiole explants within 2 weeks of inoculation in MS medium supplemented with plant growth regulators while no

Table 2 Effect of cytokinins on adventitious shoot bud regeneration from petiole-derived callus after 4 weeks of culture.

Cytokinins (μM)			% Regeneration	Mean no. of shoots/callus
BA	TDZ	Kin		
0.5			56.0±2.21e	3.2±0.23f
2.5			62.1±3.08de	5.8±0.34c
5.0			70.5±2.90c	6.4±0.52bc
7.5			65.9±3.21d	4.4±0.20e
10.0			55.3±1.20e	3.0±0.30f
	0.5		65.3±2.32d	5.9±0.45c
	2.5		78.0±2.50ab	7.0±0.60b
	5.0		85.0±4.02a	8.5±0.98a
	7.5		74.0±1.32b	6.5±0.52bc
	10.0		60.0±1.98de	4.0±0.39e
		0.5	45.7±2.95g	2.5±0.31g
		2.5	56.8±1.98e	4.0±0.62e
		5.0	61.4±2.10de	5.0±0.32d
		7.5	50.0±3.12f	3.9±0.15ef
		10.0	43.1±3.01g	2.0±0.22h

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

callus was observed in control treatment. The morphology of callus depends on the type of plant growth regulators used. Among all the concentrations of 2,4-D tested, MS medium supplemented with 5.0 μM 2,4-D was found to be most effective as it induced highest percent response (60.8±3.48) of green callus. 2,4-D resulted in high callus induction efficiency with gradual decrease as the concentration increased from 5.0 to 10.0 μM. The aforesaid callus turned morphogenic when subcultured on MS medium containing optimum concentration of 2,4-D with different concentration of TDZ (Table 1); 5.0 μM 2,4-D with 2.5 μM TDZ was found to be most effective (Fig. 2a, b) as it induced maximum percentage (81.8±4.01) of green and morphogenic callus. Our results are in contrast with Faisal et al. [9] in *Ruta graveolens* where 2,4-D and BA combinations were found to be most effective for green and nodular callus induction. Calluses were subcultured on the same medium after every 2 weeks and proliferated well.

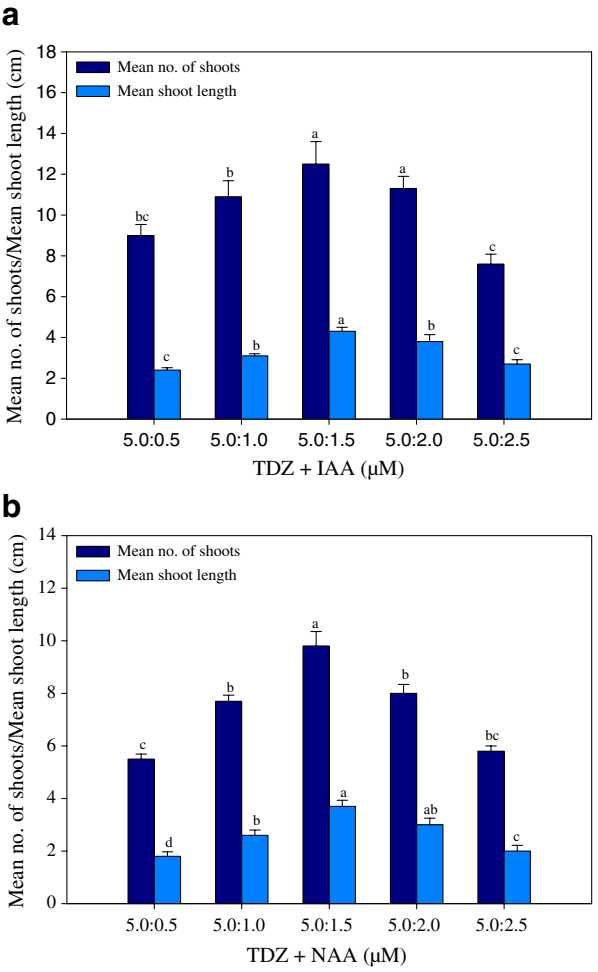

Fig. 1 Effect of TDZ and auxins (**a** IAA and **b** NAA) on in vitro shoot multiplication and shoot elongation from petiole-derived callus of *C. angustifolia*. *Bars* represent the mean ± S.E. *Bars denoted by the same letter* are not significantly different (P=0.05) by Duncan's multiple range test

Adventitious Shoot Regeneration and Shoot Elongation

Morphogenic callus induced on 5.0 μM 2,4-D and 2.5 μM TDZ when transferred to different concentrations (0.5–10.0 μM) of cytokinins, viz., BA, Kin, and TDZ, facilitated adventitious shoot bud differentiation with varying level of success (Table 2). Among the three cytokinins tested, TDZ at the concentration of 5.0 μM was found to be the most

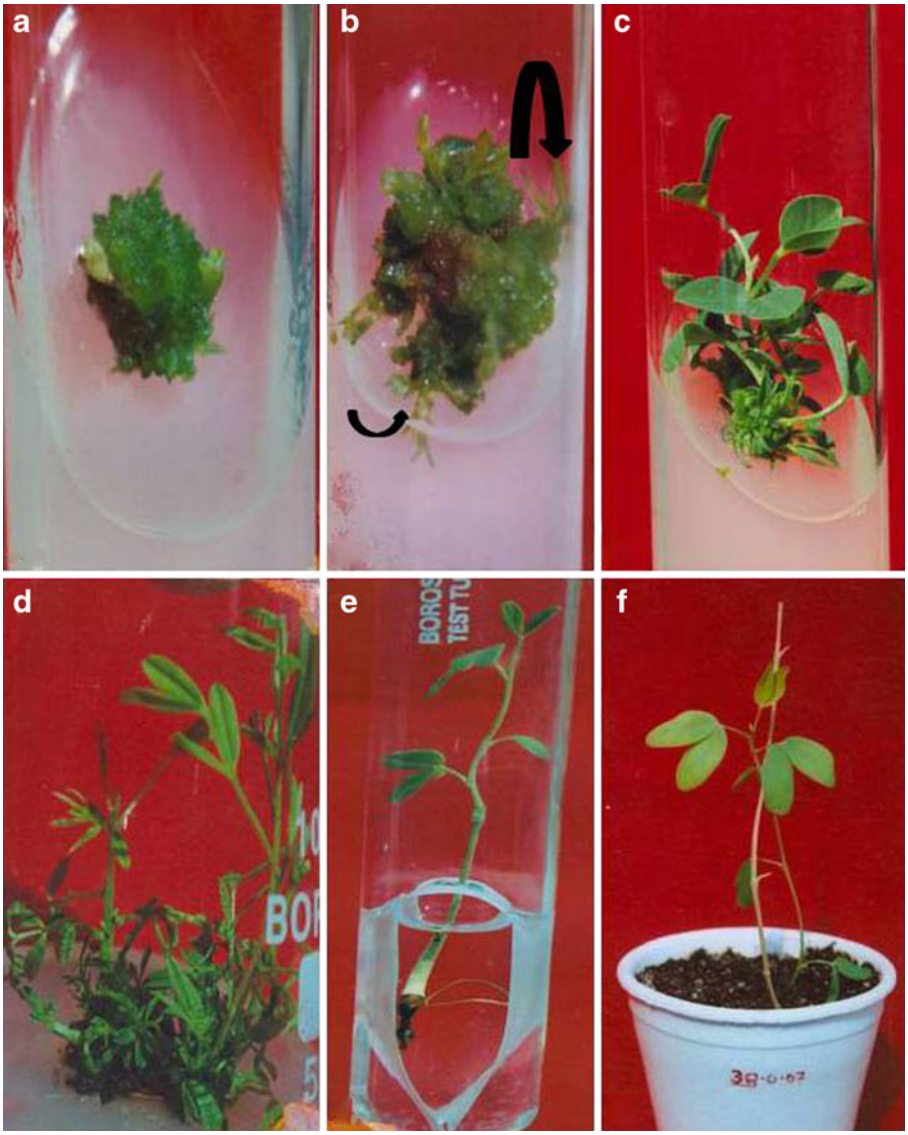

Fig. 2 Callus induction and plant regeneration from petiole explants of *C. angustifolia.* **a**, **b** Callus induction and adventitious shoot bud differentiation from petiole explants on MS medium supplemented with 2,4-D (5.0 μM)+TDZ (2.5 μM). **c** Shoot bud regeneration on MS + TDZ (5.0 μM). **d** Shoot multiplication and elongation on MS + TDZ (5.0 μM) + IAA (1.5 μM). **e** Root formation in liquid MS medium on filter paper bridge. **f** An acclimatized plant

effective for maximum adventitious shoot bud regeneration (8.5±0.98) in 85.0±4.02% cultures (Fig. 2c). At the same concentration, BA induced 6.4±0.52 shoots whereas Kin induced 5.0±0.32 shoots. The effectiveness of TDZ in shoot bud differentiation has been documented in number of plants like *Phaseolus vulgaris* [10], woody plants [11], *Bacopa monniera* [12], *Tylophora indica* [13], *C. angustifolia* [4, 5], and *Melothria maderaspatana* [14].

The Synergistic Influence of Auxins (IAA and NAA) with Cytokinin

TDZ was evident when optimal concentration of TDZ was tested with different concentration (0.5–2.5 μM) of IAA and NAA. Addition of IAA markedly enhanced the number of shoots and shoot length whereas NAA did not improve the parameters evaluated (Fig. 1a, b). Among all the TDZ-IAA and TDZ-NAA combinations tested, 5.0 μM TDZ with 1.5 μM IAA was found to be most effective combination as it produced maximum number of shoots (12.5±1.1) and shoot length (4.3±0.20 cm; Fig. 2d). A similar observation is also reported in *Feronia limonia* [15] and *C. angustifolia* [4].

Rooting of Shoots In Vitro

The microshoots of *C. angustifolia* failed to develop roots on growth regulator free MS medium. The presence of an auxin (IBA) facilitated better rhizogenesis. Rooting was carried out by two-step culture method. In the first step, 3 to 4 cm isolated microshoots were inoculated in semisolid MS medium supplemented with different concentration of IBA (0.5–12.5 μM) for 2 weeks; subsequently, they were transferred to MS liquid filter paper bridge media without IBA (second step). The maximum frequency of root formation (50.5±3.23), number of roots (3.3±0.25), and root length (3.1±0.23 cm) was recorded on MS medium supplemented with IBA (10.0 μM) after 4 weeks of culture (Table 3; Fig. 2e). The effectiveness of IBA in rooting has been reported in *T. indica* [16], *Mucuna pruriens* [17], and *Ocimum basilicum* [18]. The slow movement and slow degradation of IBA facilitated its localization near the site of application and thus its better function in inducing roots [19].

Acclimatization of Regenerated Plantlets

Plantlets with five to six leaves and well-developed root system were removed and transferred to pots containing sterile soilrite for 4 weeks (Fig. 2f) and eventually established

Table 3 Effect of different concentrations of IBA on root induction from in vitro raised microshoots of *C. angustifolia* after 4 weeks of culture.

IBA (μM)	% Rooting	Mean no. of roots/shoot	Mean root length (cm)
0.5	NR	NR	NR
2.5	38.7±1.98c	1.7±0.20c	1.8±0.20c
5.0	44.8±2.59b	2.5±0.13b	2.2±0.18bc
10.0	50.5±3.23a	3.3±0.25a	3.1±0.23a
12.5	40.5±1.52c	2.0±0.40bc	2.5±0.18b

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

in natural soil. Seventy percent of the plantlets survived during acclimatization and acclimatized plants were transferred to greenhouse. The regenerated plants did not show detectable variation in morphological or growth characteristics compared to the parent plant.

Conclusion

An efficient protocol for the micropropagation of *C. angustifolia* via callus induction and adventitious multiple shoot bud differentiation and proliferation has been standardized. The micropropagated plants were successfully acclimatized in greenhouse and were similar to the donor plants in their morphology. The protocol established here can serve as an efficient method for the rapid and large-scale production of this potential medicinal plant from which useful secondary metabolite "sennosides" are extracted.

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