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BRIEF COMMUNICATION

In vitro propagation of *Cassia angustifolia* through leaflet and cotyledon derived calli

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Abstract

High efficiency shoot regeneration was achieved through leaflet and cotyledon derived calli in *Cassia angustifolia* - an important medicinal plant. Dark brown compact callus was induced at the cut ends of the explants on Murashige and Skoog's (MS) medium augmented with 1 μM N^6-benzyladenine (BA) + 1 μM 2,4-dichlorophenoxyacetic acid (2,4-D). Such callus pieces on transfer to cytokinins (BA or kinetin) supplemented medium differentiated shoots within 10 - 15 d. Of the two cytokinins, 5 μM BA was optimum for eliciting morphogenic response in 83.33 and 70.83 % cultures with an average of 4.16 ± 0.47 and 3.70 ± 0.56 shoots in cotyledon and leaflet derived calli, respectively. The addition of 0.5 μM α-naphthaleneacetic acid (NAA) to MS + 5 μM BA further elevated the maximum average number of shoots to 12.08 ± 1.04 and 5.37 ± 0.52 for cotyledon and leaflet calli, respectively. The excised shoots were transferred to a rooting medium containing either IAA (indole-3-acetic acid), IBA (indole-3-butyric acid) or NAA. Nearly 95 % shoots developed an average of 5.4 ± 0.41 roots on half strength MS medium supplemented with 10 μM IBA.

Additional key words: benzyladenine, indole-3-butyric acid, multiple shoots, naphthaleneacetic acid, rooting, senna.

Cassia angustifolia Vahl, commonly known as senna, is a medicinally valuable drought resistant shrub of family *Leguminosae*. It is a native of Saudi Arabia and has been naturalized in India. The plant has also been recommended for developing wastelands and does not require frequent irrigation.

Though the species which belong to the *Leguminosae* are known for their recalcitrant nature, yet some successful attempts have been made on *in vitro* organogenesis of *Cassia fistula, C. siamea* (Gharyal and Maheshwari 1990) and *C. alata* (Fett *et al.* 2000). Incidentally, there is no report on *in vitro* regeneration of *C. angustifolia* except our earlier paper (Agrawal and Sardar 2003) on seedling derived explants. Reports on its cultivation and genetic improvement programme are also Iimited (Jambhale *et al.* 1998, Lal *et al.* 1992). Regeneration *via* calli has been the potent source of producing somaclonal variants in plants and thus the improvement of the species. Many plants have been propagated through cotyledon and leaf derived calli, *e.g. Cardiospermum halicacabum* (Babber *et al.* 2001), *Vigna radiata* (Amutha *et al.* 2003) and *Melia azedarach* (Vila *et al.* 2003/4). The aim of the present study was to develop a callus mediated plant regeneration system for C. *angustifolia.*

Seeds procured from Central Arid Zone Research Institute, Jodhpur, Rajasthan, India, were used as the experimental material. Thorough washing of seeds was done under running tap water for 20 - 25 min followed by overnight soaking in water. The seeds were surface sterilized using 0.1 % $HgCl_2$ solution for 5 - 10 min and then rinsed 4 or 5 times with sterile distilled water and inoculated on MS basal medium for germination, at temperature of 25 ± 2 °C under cool white fluorescent tubes (*Philips India Ltd*., Kolkata, India) with continuous irradiation of approximately 45 - 46 W m^{-2}. Seedlings, 20-d-old, thus raised, were employed as explants.

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Abbreviations: BA - N^6-benzyladenine; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; NAA - α-naphthaleneacetic acid; 2,4-D - 2,4-dichlorophenoxyacetic acid; Kn - kinetin; MS - Murashige and Skoog (1962) medium.

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Murashige and Skoog's (1962; MS) basal medium supplemented with 3 % (m/v) sucrose (*DCM*, Daurala, India) and 0.8 % agar (*Qualigens*, Mumbai, India) was used in all the experi-ments. The pH was adjusted to 5.8 using 0.1 M NaOH or 0.1 M HCl prior to autoclaving. Routinely, 25 cm^3 molten medium was dispensed in each 25 × 150 mm culture tube (*Borosil Glass Works*, Mumbai, India), plugged with non-absorbent cotton and sterilized at pressure of 1.06 kg cm^{-2} for 15 min. All cultures were incubated at 25 ± 2 °C, under continuous light with irradiance of approximately 45 - 46 W m^{-2} (cool fluo-rescent tubes, *Philips*). Cotyledon and leaflet explants were cultured on MS medium augmented with 1 μM 2,4-dichlorophenoxyacetic acid (2,4-D) in combination with 1 μM N^6-benzyladenine (BA) to induce callus. For initiation of shoots, the calli were subcultured on MS medium containing different concentrations (0, 0.5, 1, 5, and 10 μM) of BA or kinetin (Kn) either alone or in combinations with 0.5 μM α-naphthalene-acetic acid (NAA). For rooting, shoots (3 - 3.5 cm long) were excised and transferred to half-strength MS medium with 2 % (m/v) sucrose without growth regulators or supplemented with 0.5 - 10 μM IBA, NAA or IAA. All chemicals used were of analytical grade (*Sigma-Aldrich* and *E. Merck*).

After removing the plantlets from semisolid medium, they were washed thoroughly with autoclaved water and dipped for 15 min in 0.1 % (m/v) *Bavistin* (*BASF*, Mumbai, India). They were initially transplanted in plastic pots with *Soilrite* for one month. The pots were covered with polythene sheets and maintained under controlled conditions (45 W m^{-2}, 25 ± 2 °C, 55 - 65 % RH). The plantlets were irrigated with ¼ strength MS solution for one week and then with tap water. Later on, they were transferred to soil.

Cultures were observed on visual basis daily and weekly using stereobinocular (*Olympus*, Japan). The result presented are the means of 2 replicates of 24 explants ± SE.

Tissues for histological examinations at different developmental stages were fixed in FAA [formalin/glacial acetic acid/ethanol (70 %) in the ratio of 5:5:90 (v/v)] for 12 h, dehydrated through graded ethanol-xylol series and embedded in paraffin wax. The embedded tissues were cut with a microtome at 8 μm thickness, stained with hematoxylin-eosin, mounted with *p*-xylenebispyridinium bromide (DPX, *Qualigens*), and observed microscopically.

Initially, dark brown compact callus developed at cut ends of cotyledon and leaflet explants from 20-d-old *in vitro* grown seedlings within 7 - 10 d of inoculation on Murashige and Skoog's medium augmented with 1 μM 2,4-D in combination with 1 μM BA. The callus proliferated rapidly and subsequently covered the entire surface of the explants (Fig. l*A*). The aforesaid dark brown compact callus turned greenish and morphogenic when subcultured on media containing different concentration (0, 0.5, 1, 5, and 10 μM) of BA or Kn alone or in combination with NAA (0.5 μM). Histological observations revealed the formation of meristemoids (Fig. 1*B*) which subsequently organized shoot primordia (Fig. 1*C*) in the parenchymatous tissue of callus cultured on MS + 1 μM BA. In next 10 to 15 d such shoot primordia developed into shoots (Fig. 1*D*). Of the two

Table 1. Effect of BA, Kn and NAA on the morphogenic response of cotyledon and leaflet derived calli of *Cassia angustifolia* reared on MS medium for 30 d. Means ± SE, *n* = 24.

BA [μM]	Kn [μM]	NAA [μM]	Cotyledon response [%]	shoot number [explant^{-1}]	shoot length [cm]	Leaflet response [%]	shoot number [explant^{-1}]	shoot length [cm]
0.5			62.50	1.33 ± 0.23	1.68 ± 0.35	54.16	1.08 ± 0.22	1.17 ± 0.25
1.0			70.83	3.58 ± 0.54	3.07 ± 0.92	62.50	2.25 ± 0.40	1.81 ± 0.48
5.0			83.33	4.16 ± 0.47	3.15 ± 0.76	70.83	3.70 ± 0.56	2.12 ± 0.60
10.0			58.33	3.20 ± 0.59	1.43 ± 0.47	41.60	1.91 ± 0.49	1.43 ± 0.50
0.5		0.5	54.16	1.00 ± 0.20	2.14 ± 0.43	37.50	0.91 ± 0.26	1.60 ± 0.42
1.0		0.5	75.00	4.08 ± 0.57	3.43 ± 0.91	75.00	3.25 ± 0.59	1.98 ± 0.48
5.0		0.5	91.66	12.08 ± 1.04	3.30 ± 0.95	87.50	5.37 ± 0.52	2.56 ± 0.54
10.0		0.5	66.66	3.83 ± 0.61	1.67 ± 0.54	54.16	2.16 ± 0.47	1.53 ± 0.46
	0.5		45.83	1.20 ± 0.30	1.32 ± 0.36	45.83	0.87 ± 0.21	1.35 ± 0.29
	1.0		62.50	3.33 ± 0.56	2.95 ± 0.91	45.83	2.04 ± 0.49	1.62 ± 0.54
	5.0		75.00	3.45 ± 0.45	3.06 ± 0.74	66.66	3.29 ± 0.53	2.13 ± 0.61
	10.0		50.00	2.41 ± 0.53	1.46 ± 0.50	33.33	1.83 ± 0.56	1.24 ± 0.50
	0.5	0.5	45.83	0.95 ± 0.25	1.80 ± 0.45	29.16	0.62 ± 0.20	1.02 ± 0.27
	1.0	0.5	75.00	3.08 ± 0.42	3.13 ± 0.71	66.66	2.91 ± 0.46	1.76 ± 0.46
	5.0	0.5	87.50	4.41 ± 0.47	3.18 ± 0.68	75.00	4.62 ± 0.60	2.34 ± 0.65
	10.0	0.5	54.16	3.29 ± 0.65	1.25 ± 0.45	37.50	1.95 ± 0.58	1.23 ± 0.49

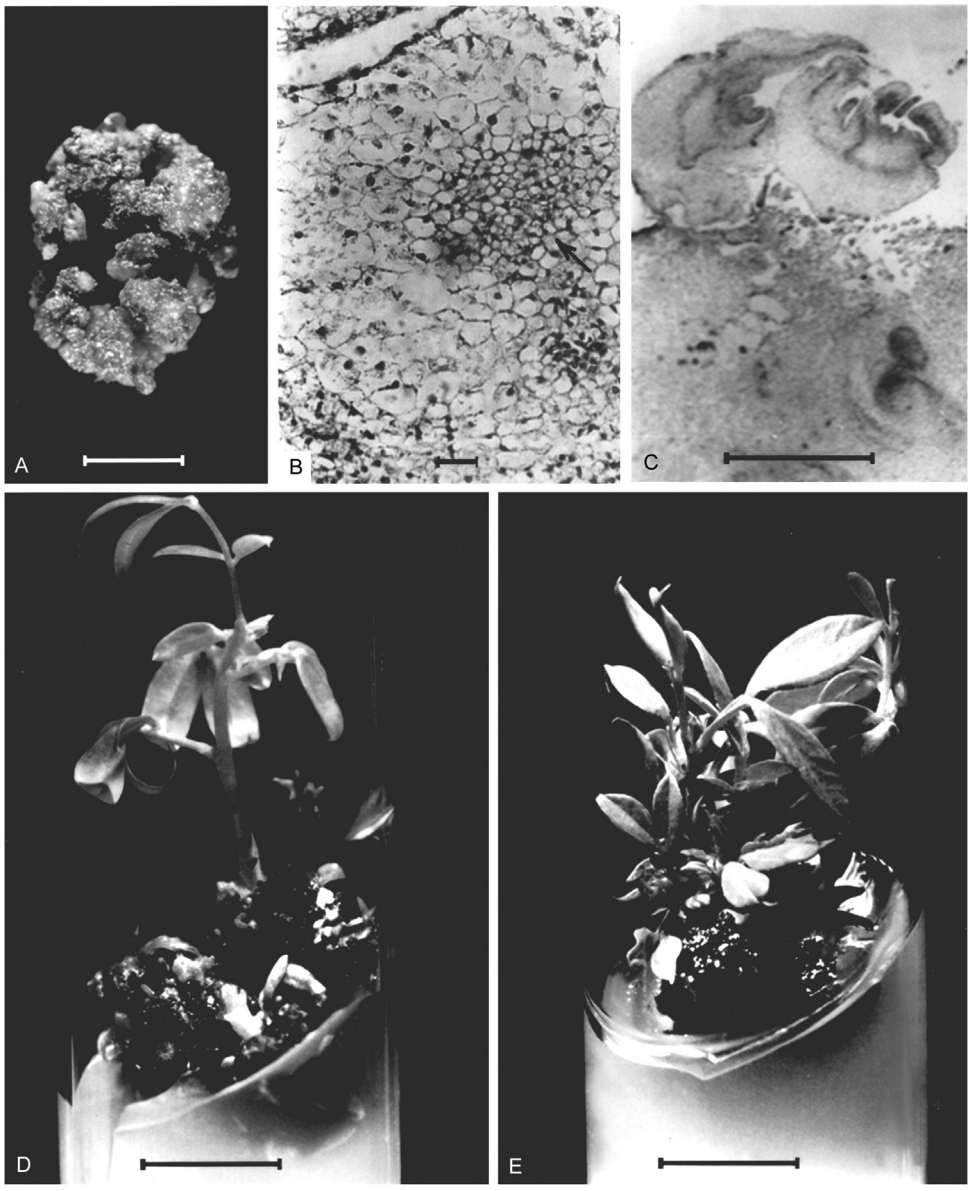

Fig. 1. *In vitro* regeneration from callus of senna (*Cassia angustifolia*). *A* - dark brown compact callus derived from cotyledon explant cultured on 1 μM 2,4-D + 1 μM BA (*bar* = 1 cm); *B* - callus cultured on MS + 1 μM BA showing a meristemoid (*arrow*) surrounded by parenchymatous tissue (*bar* = 100 μm). *C* - induction of shoot buds in the callus subcultured on MS + 1 μM BA after one week (*bar* = 1 mm); *D* - formation of a shoot and multiple shoot buds from cotyledon derived callus subcultured on MS + 1 μM BA after 15 d (*bar* = 1 cm); *E* - differentiation of multiple shoots from cotyledon derived callus subcultured on MS + 5 μM BA + 0.5 μM NAA after 30 d (*bar* = 1 cm).

cytokinins, BA or Kn the former at 5 μM was optimum for eliciting morphogenic response in 83.33 and 70.83 % cultures with an average of 4.16 ± 0.47 and 3.70 ± 0.56 shoots in cotyledon and leaflet derived calli, respectively. A further improvement in shoot regeneration was achieved using combinations of BA with NAA (Table 1). The addition of 0.5 μM NAA to 5 μM BA augmented MS medium resulted in maximum number of shoots (12.08 ± 1.04) for cotyledon callus (Fig. 1*E*), while leaflet callus exhibited a low frequency of shoot formation (Table 1), developing an average of 5.37 ± 0.52 shoots. These results on the synergistic effect of NAA in combination with cytokinin are in consonance with the findings of Angeloni *et al.* (1992) on *Centrosema brasilianum*, Sudha and Seeni (1996) on *Rauwolfia micrantha* and Zhao *et al.* (2003/4) on *Sophora flavescens.* Incidentally, during present investigation the synergistic effect of Kn in combination with NAA (0.5 μM) for both the callus types did not exhibit significant increase in the morphogenic response

(Table 1). Contrary to this, in quince leaves Kn + NAA treatment caused substantial modification in the proportions among the groups of regenerating leaves with increasing treatment length (D'Onofrio and Morini 2003/4). Higher concentration of cytokinins (10 μM) either alone or in combination with NAA did not favour elongation of shoots in both the cotyledon and leaflet derived callus. The effects of explant source and growth regulators on callus-mediated organogenesis exhibited significant differential response in terms of percentage morphogenic cultures, average shoot number and average shoot length.

It is thus well documented that callus derived from cotyledon was more responsive than that from leaflet and among the combinations of cytokinins with auxin, BA + NAA showed higher regeneration potential compare to Kn + NAA for both callus types. In *Cassia angustifolia*, shoot regeneration potential in the callus derived from cotyledon and leaflet persisted for more than 2 years.

Well developed shoots (3 - 3.5 cm long) from the culture were excised and transferred to rooting medium containing half strength MS salts augmented with IBA, NAA or IAA. Basal medium did not support the induction of roots. However, 95 % of shoots organised an average of 5.4 ± 0.41 roots on MS (½) + 10 μM IBA medium (Table 2, Fig. 2*A*). These results are in agreement with the finding of Dhar *et al.* (2000) on *Pittosporum napaulensis* where IBA was found better than NAA and IAA to induce the formation of maximum number of roots. The slow movement and slow degradation of IBA might facilitate its localization near the site of application and thus its better function in inducing roots (Martin 2002). IBA also proved effective in inducing rooting in *Simmondsia chinensis* (Agrawal *et al.* 2002). The plantlets, thus raised, were transfer to *Soilrite*. They were maintained there for hardening and subsequently transferred to soil for further acclimatization (Fig. 2*B*).

Table 2. Rooting response of *in vitro* raised shoots of *C. angustifolia* to auxins supplemented half strength MS medium after 30 d of subculture. Means ± SE, *n* = 24.

Auxins	[μM]	Shoots with roots [%]	Root number [shoot^{-1}]	Root length [cm]
IBA	0.5	60	0.95 ± 0.19	1.17 ± 0.24
	1.0	70	2.15 ± 0.37	1.88 ± 0.55
	5.0	85	3.50 ± 0.40	4.06 ± 0.88
	10.0	95	5.40 ± 0.41	4.65 ± 0.70
NAA	0.5	55	0.75 ± 0.17	2.17 ± 0.46
	1.0	60	1.95 ± 0.38	3.08 ± 0.90
	5.0	70	2.85 ± 0.47	3.56 ± 0.98
	10.0	85	3.95 ± 0.45	3.04 ± 0.84
IAA	0.5	50	0.65 ± 0.16	1.65 ± 0.34
	1.0	55	1.50 ± 0.33	2.12 ± 0.59
	5.0	65	2.30 ± 0.42	3.21 ± 0.90
	10.0	80	3.05 ± 0.40	2.36 ± 0.55

In conclusion, an efficient protocol has been developed for mass propagation from callus cultures of an important medicinal plant *Cassia angustifolia.* The *in vitro* technique may be helpful for sustainable supply of raw material to the pharmaceutical industries and selection of somaclonal variants that may be used to widen the genetic base of this genus and genetic transformation through *Agrobacterium* co-cultivation to recover transgenic plants.

Fig. 2. *A* - induction of roots in the excised *in vitro* raised shoot of senna *(Cassia angustifolia* Vahl) on half strength MS + 10 μM IBA after 20 d of transfer (*bar* = 1 cm); B - two month-old tissue culture-derived plantlet thriving in soil (*bar* = 1 cm).

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