Micropropagation and validation of genetic and biochemical fidelity amongst regenerants of *Cassia angustifolia* Vahl employing RAPD marker and HPLC

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Abstract *In vitro* protocol has been established for clonal propagation of *Cassia angustifolia* Vahl which is an important source of anticancerous bioactive compounds, sennoside A and B. Nodal explants excised from field raised elite plant (showing optimum level of sennoside A and B) of *C. angustifolia* when reared on Murashige and Skoog's medium augmented with different cytokinins, viz. N⁶-benzyladenine (BA), N⁶-(2-isopentenyl) adenine (2iP) and 6-furfuryl aminopurine (Kn) differentiated multiple shoots in their axils. Of the three cytokinins, BA at 5 µM proved optimum for differentiating multiple shoots in 95 % cultures with an average of 9.14 shoots per explant within 8 weeks of culture. Nearly, 95 % of the excised *in vitro* shoots rooted on half strength MS medium supplemented with 10 µM indole-3-butyric acid (IBA). The phenotypically similar micropropagated plants were evaluated for their genetic fidelity employing random amplified polymorphic DNA (RAPD) markers. Eleven individuals, randomly chosen amongst a population of 120 regenerants were compared with the donor plant. A total of 36 scorable bands, ranging in size from 100 to 1,000 bp were generated amongst them by the RAPD primers. All banding profiles from micropropagated plants were monomorphic and similar to those of mother plant proving their true to the type nature. Besides, high performance liquid chromatography evaluation of the sennoside A and B content amongst leaves of the mature regenerants and the elite mother plant too revealed consistency in their content.

Keywords *Cassia angustifolia* Vahl · Cytokinin · Genetic Fidelity · Micropropagation · RAPD · Sennoside

Abbreviations

CTAB	Cetyl trimethyl ammonium bromide
DMRT	Duncan's multiple range test
f.w.	Fresh weight
HPLC	High performance liquid chromatography
ISSR	Inter simple sequence repeat
MS	Murashige and Skoog
PDA	Photo Diode Array
RAPD	Random amplified polymorphic DNA
SPSS	Statistical package for social sciences

Introduction

Cassia angustifolia Vahl, a native of Saudi Arabia commonly known as senna, is a medicinally valuable drought-resistant shrub of family Leguminosae mainly grown as a cash crop in different parts of the world. Leaves and pods of senna are chief source of important anthraquinone glycosides known as sennosides (Council of Scientific Industrial Research 1950), which are extensively employed as a laxative in pharmaceutical industry. Besides being an excellent laxative, senna is also used as a febrifuge in splenic enlargements, cholera, jaundice, gout, rheumatism, foul breath, etc. (Pulliah 2002). Sennoside A and B, the major bioactive compounds of *C. angustifolia* are optical isomers having the chemical formulae, C₄₂H₃₈O₂₀ and a molecular weight of 862.75. Both sennoside A and B are reported to have significant bioefficacy against various cancer cell lines (Waltenberger et al. 2008; Chen et al. 2009; Chang et al. 2012). Thus, sennosides may have significant potential for anti-cancer therapy.

India's exports in traditional healthcare products besides Indian Systems of Medicine (ISM), includes significant portion of medicinal plants and extracts and as per Export Import (EXIM) bank (2010) report, senna leaves are included as

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major items of medicinal plants and extracts that are being exported from India. Considering the potential global economic importance of this species, there is a need for large scale production of quality planting materials for raising commercial plantations. *Senna* is a potential crop for honeybee culture, as its flowers provide food supply for honeybee throughout a year (Sharma et al. 1999). Since the plant is eco-friendly, it has been recommended for restoring wastelands, as it does not require frequent irrigation (Bohra and Sankhla 1997). Its drought resistance, quick establishment and perennial nature provide permanent green cover on sand in vegetation-deficient arid zones.

Due to its multifold uses, the plant is being highly exploited by the pharmaceutical companies and it has therefore, become imperative to develop a protocol for mass propagation through tissue culture for large scale production of sennosides and their further elevation using elicitors and through genetic transformation. Though, some work on *in vitro* regeneration of *C. angustifolia* has been published (Agrawal and Sardar 2003, 2006, 2007; Siddique et al. 2010; Parveen and Shahzad 2011; Parveen et al. 2012) using different explants, none of them have established protocols pertaining to genetic and biochemical fidelity of the mature regenerants. Since the *in vitro* plant regeneration can cause genetic instability and somaclonal variation due to culture stress (Haisel et al. 2001), the direct development of a plant regeneration system and assessment of clonal fidelity of the *in vitro* raised plants of *Cassia angustifolia* will be of great significance in this crop. Application of molecular markers such as RAPD, ISSR, etc. to the micropropagated plants have proved beneficial for analysing the genetic fidelity (*Gerbera jamesonii*, Bhatia et al. 2011; *Celastrus paniculatus*, Phulwaria et al. 2012; *Eclipta alba*, Singh et al. 2012, etc.). Besides, assessment of consistency in sennoside A and B content from mature regenerated plants vis-à-vis elite donor plants is also an important parameter for assessment of biochemical fidelity amongst the *in vitro* raised mature regenerants of *C. angustifolia*. Present investigation, highlights the efficient protocol for rapid clonal multiplication of *C. angustifolia* Vahl through nodal explants and establishment of genetic and biochemical fidelity amongst the micropropagated plants.

Materials and methods

Plant material

Comprises nodal explants excised from field grown 6-month-old elite plant of *C. angustifolia* Vahl (Fig. 1a). Such plants were raised through seeds (procured from Jodhpur, India) in the Botanical Garden, University of Delhi, Delhi. The elite plant was selected among 30 plants on the basis of random sampling for sennoside A and B content employing HPLC

Fig. 1 Direct organogenesis through nodal explants of *Cassia angustifolia* Vahl after 8 weeks of culture. **a** Field raised elite plant of *C. angustifolia* (inset shows nodal explant at the time of inoculation). **b** & **c** Nodal explant on MS medium supplemented with 0.5 μ M BA and 10 μ M 2iP, respectively. **d** Differentiation of multiple shoots from nodal explant after 8 weeks of culture on MS+5 μ M BA. **e** Rooting of *in vitro* raised shoot on MS (1/2)+10 μ M IBA after 2 weeks. **f** Tissue culture-derived plant acclimatized to soil in glass house. **g** Four month old tissue culture raised plants in garden

(data not shown here). The nodal explants of the micro shoots raised in the axils of nodes of the aforesaid elite plant were employed for conducting different experiments. The leaves of aforesaid mature *ex vitro* regenerated plants were collected separately for analysis of uniformity in sennoside A and B content.

Surface sterilization of explants

The excised nodal explants of *C. angustifolia* were immediately dipped in freshly prepared 1 % (w/v) citric acid (SRL, Mumbai, India) solution to minimize browning. Surface

sterilization was done by treating the explants with 5 % (v/v) teepol (detergent; Rickett and Colman India Ltd., Kolkata, India) and 0.5 % (w/v) bavistin solution (a systemic fungicide; BASF India Ltd., Mumbai, India) each for 15 min, followed by 70 % (v/v) ethanol for 5 min, freshly prepared 0.1 % HgCl_2 (SRL, Mumbai, India) and 150 mg/L cefotaxime solution for 5 min each with continuous shaking under a laminar flow cabinet. The nodal explants were finally washed four or five times with sterile distilled water on a laminar flow cabinet prior to implantation in semisolid media.

Culture medium and growth conditions

The Murashige and Skoog 1962 (MS) basal medium was supplemented with different concentrations (0, 0.5, 1, 5 and 10 μM) of N^6 -benzyladenine (BA) or N^6 -(2-isopentenyl) adenine (2iP) or 6-furfuryl aminopurine (Kn). For rhizogenesis, half strength MS (Murashige and Skoog 1962) basal medium with α -naphthalene acetic acid (NAA) or indole-3-butyric acid (IBA) or indole-3-acetic-acid (IAA) at different concentrations (0, 0.5, 1, 5 and 10 μM) were employed. All plant regulators were obtained from Sigma Aldrich (USA). Salts and other chemicals were obtained from Qualigens, Glaxo and SRL, Mumbai (India). As carbon source, 3 % (w/v) sucrose (DCM, Daurala, India) was added to the media and the pH was adjusted to 5.8 using 0.1 N NaOH or 0.1 N HCl. Approximately, 20 ml media was dispensed in each 150×25 cm test tubes (Borosil, India), plugged with non-absorbent cotton wrapped in two-layered muslin cloth and sterilized by autoclaving at 1.06 kg cm^{-2} at 121°C for 15 min. The nodal explants were cultured in test tubes containing semisolid medium in a vertical orientation. Cultures were maintained on continuous light ($450\text{--}460 \mu\text{W cm}^{-2}$) by cool day light emitted from fluorescent incandescent tubes (40 W, Philips, Kolkata, India) of 16 h light followed by 8 h dark photo period. The cultures were maintained in a culture room at $25 \pm 2^\circ\text{C}$ temperature with a relative humidity of 55 ± 5 %.

Acclimatization of regenerated plantlets

After rhizogenesis, healthy plantlets with well developed roots were removed from medium and were washed under running tap water to remove the adhering medium. They were treated with 1 % bavistin (BASF, Mumbai, India) solution to prevent any fungal infection, before being transferred to plastic pots (5 cm diameter) containing autoclaved soil. Subsequently, acclimatization was achieved by covering the plastic pots with polythene bags with 4–8 holes to maintain the humidity and the plants were irrigated with one-tenth of major salt solution of MS medium after every 4 days. The potted plants were maintained inside a culture room. After 6 weeks, the plantlets were transplanted to pots containing garden soil and kept

under shade in green house for another 2 weeks before being transferred to field for developing into mature plants.

Phytochemical analysis

HPLC analysis for the estimation of sennoside A and B was performed following the protocol modified from Bala et al. (2001). The solvent system consisted of solvent A: methanol + water + acetic acid (20:80:0.1) and solvent B: methanol + water + acetic acid (80:20:0.1). The HPLC unit (Waters, USA) equipped with PDA (Photo Diode Array) detector and printer plotters was operated under the following parameters: column: C18; column packing: Zorbex ODS (Octadecyl silane); solvent: methanol (HPLC grade); injection volume: 20 μl ; flow rate: 0.5 ml/min; detection: UV 285 nm for sennoside A and B. Elution was carried out starting at 80 % solvent A for 5 min, changing to 100 % solvent B over 20 min, isocratic at 100 % solvent B for 10 min and then 80 % solvent A for 5 min.

DNA isolation and RAPD analysis

For genetic fidelity studies, 11 field transferred mature regenerated plants were chosen randomly amongst a population of 120 mature field transferred regenerated plants along with the elite donor mother plant of *C. angustifolia*. Total genomic DNA of donor mother plant and the *in vitro* raised clones of *C. angustifolia* were extracted from young leaf tissue (2 g) by using the Cetyl trimethyl ammonium bromide (CTAB) method as described by Saghai-Marooof et al. (1984). A set of 20 decamer RAPD (Sigma Aldrich, Bangalore, India) primers were screened for their repeatable amplification with the DNA from the aforesaid plants including the elite mother plant to assess the genetic stability of the regenerants. The number of RAPD primers selected for the analysis was done on the basis of their amplification products for clear and scorable banding patterns. RAPD amplification was carried out in a total volume of 25 μl containing 20 ng of genomic DNA, 2.5 μl of 10X PCR buffer containing 15 mM MgCl_2 , 0.2 mM dNTPs, 1 unit Taq polymerase (Bangalore Genei, India) and 20 ng RAPD primer (Sigma Aldrich, USA). Amplification conditions using RAPD primers were performed as initial DNA denaturation at 94°C for 4 min, followed by 45 cycles of 1 min denaturation at 94°C , 1 min annealing at 36°C and 2 min of extension at 72°C with a final extension at 72°C for 7 min. The amplified DNA fragments were separated on a 1.2 % (w/v) agarose gel using 0.5X TBE buffer and stained with ethidium bromide (0.5 $\mu\text{g/ml}$). The size of the amplicons was estimated by comparing with 1 Kb DNA ladder (Bangalore Genei, India). Gels were visualized and photographed by an Alpha Imager Gel Documentation System (Alpha Innotech Corporation, USA).

Recording of data and statistical analysis

The morphogenic response of explants for *in vitro* regeneration was evaluated after 8 weeks of culture in terms of (1) percentage of explants producing single shoot or multiple shoots, (2) average shoot number per explant and (3) average shoot length per explant. For rhizogenesis, following parameters were considered: (1) percentage of shoots developing roots, (2) average number of roots per shoot and (3) average root length. The average number of shoots per explant, the average shoot length, the average number of roots per shoot and the average root length has been represented as mean values along with standard error (mean±SE). The mean values were calculated on the basis of a minimum of 24 replicates in each experiment and repeated at least for once or twice. The evaluation of sennoside A and B from the different plant parts of *C. angustifolia* was performed with four replicates. The data expressed as mean±SE have been statistically analyzed using Analysis of Variance (ANOVA) through Statistical Package for Social Sciences (SPSS) version 16.0. The differences between means were tested for significance by Duncan's multiple range test (DMRT) at $p=0.05$.

For genetic fidelity studies, amplified DNA bands were recorded with all the selected RAPD primers and only clear and scorable bands at a particular position were considered. Each band was treated as a marker and scoring of bands were done on the basis of their presence ('1') or absence ('0') in the gel. Intensity of the band was not considered while scoring.

Results

Induction of multiple shoots and elongation

The nodal explants of *C. angustifolia* when inoculated on MS medium supplemented with various concentrations (0, 0.5, 1, 5 and 10 μM) of different cytokinins (BA, KN and 2iP) showed differential morphogenic responses. Basal medium did not support the induction of multiple shoots even after 8 weeks of culture (Table 1). Differentiation of multiple shoots occurred only on cytokinin enriched medium. Of the aforesaid three cytokinins tried, BA proved most effective in differentiating multiple shoots. At 5 μM BA, there was bud break in the axil which proliferated further and developed multiple shoots in the next 2 weeks. Nearly, 95.12 % cultures developed multiple shoots with an average of 9.14 shoots per explant having an average shoot length of 3.55 cm after 8 weeks of culture (Table 1, Fig. 1d). Lower concentration (0.5 μM) of BA displayed poor morphogenic response with an average of 1.59 shoots (Table 1, Fig. 1b). At higher concentration (10 μM) of BA, induction of multiple shoots as well as average shoot length were seen to be decreased with an average of 4.63 shoots (Table 1). Besides, initiation of greenish black callus was observed at the base of explants at higher concentration of BA. Contrary to BA, all the tried concentrations of Kn or 2iP displayed poor response in terms of percentage responding cultures inducing multiple shoots as well as average number of induced shoots and shoot length (Table 1; Fig. 1c). The optimum morphogenic response of Kn and 2iP in terms of average number of shoots was 1.14

Table 1 Effect of cytokinins supplemented to MS medium on shoot induction from nodal explants of *Cassia angustifolia* Vahl. Experiment was repeated once bearing consistent results

Cytokinins (μM)	% of cultures inducing multiple shoots*	Average no. of shoots* (mean±SE)	Shoot length (cm) * (mean±SE)
Control			
0	—	—	—
BA			
0.5	56.28 ^c	1.59±0.008 ^h	2.78±0.007 ^h
1.0	60.44 ^d	2.37±0.006 ⁱ	1.32±0.011 ^f
5	95.12 ⁱ	9.14±0.006 ^k	3.57±0.008 ⁱ
10	64.27 ^f	4.63±0.008 ^j	0.67±0.007 ^a
Kn			
0.5	52.13 ^b	0.53±0.006 ^b	0.75±0.010 ^b
1.0	62.54 ^e	0.85±0.007 ^c	0.91±0.008 ^d
5	68.76 ^h	1.14±0.009 ^g	1.45±0.007 ^g
10	56.27 ^c	0.62±0.007 ^c	0.68±0.006 ^a
2-iP			
0.5	41.68 ^a	0.45±0.009 ^a	0.74±0.007 ^b
1.0	52.13 ^b	0.84±0.008 ^c	0.80±0.007 ^c
5	68.76 ^h	1.07±0.006 ^f	1.24±0.007 ^c
10	64.61 ^g	0.76±0.008 ^d	0.66±0.106 ^a

*Mean values in a column followed by different letters are significantly different as determined by SPSS at $p=0.05$ according to DMRT

shoots at 5 μM Kn and 1.07 shoots at 5 μM 2iP (Table 1). However, such cultures did not show improved growth even on subculturing (Table 1; Fig. 1c).

Induction of roots and hardening of plantlets

The excised *in vitro* shoots were transferred to half strength MS (Murashige and Skoog 1962) basal medium containing different concentrations (0, 0.5, 1, 5 and 10 μM) of three auxins namely α -naphthalene acetic acid (NAA) or indole-3-butyric acid (IBA) or indole-3-acetic-acid (IAA). Among the three, IBA was the most effective. A maximum of 91.6 % shoots induced an average of 5.14 roots with an average root length of 4.76 cm after 3 weeks on half strength MS medium augmented with 10 μM IBA (Table 2; Fig. 1e). The roots were induced directly from the shoot base without callus formation at this concentration. None of the shoots induced rooting on basal medium. Compared to IBA, poor rooting response was observed with all the tried concentrations of NAA and IAA (Table 2). The tissue culture-derived plantlets (Fig. 1f) were acclimatized in field with 95 % survival. Such micropropagated plants were found to be phenotypically similar with the mother plant (Fig. 1g) and their genetic as well as biochemical fidelity was assessed using RAPD and HPLC analysis, respectively.

Genetic fidelity using RAPD analysis

Genetic fidelity of micropropagated plants of *C. angustifolia* was evaluated employing PCR based fingerprinting

technique. RAPD analysis was done by randomly selecting 11 chosen micropropagated plants vis-a-vis the mother plant. A total of 20 arbitrary decamers (RAPD primers) were used for initial screening out of which 10 produced a total of 36 clear, distinct and reproducible scorable bands with an average of 4.3 bands per primer with size ranging from 100 to 1,000 bp (Table 3). All the banding profile from micropropagated plants were monomorphic and similar to those of mother plant except two primers, viz. VAB-06 and VAB-10 which displayed polymorphic bands in two of the regenerants (Table 3). The RAPD primer VAB-05 displayed prominent monomorphic bands amongst the *in vitro* derived regenerants and the elite donor mother plant (Fig. 2a), while the primer VAB-10 displayed polymorphic band in one of the regenerants (Fig. 2b).

HPLC analysis for sennoside A and B content of mature regenerants and mother elite plant of *C. angustifolia*

The quantitative analysis of sennoside A and B content of different plant parts viz. seeds, leaves, nodes and roots of field raised plant of 4 month old garden raised plant of *C. angustifolia* through HPLC revealed that the amount of sennoside A and B was variable in the different parts but was maximum in leaves (Figs. 3a–f and 4a). The respective concentrations of sennoside A and B were 2,861.14 $\mu\text{g/g}$ f.w. and 250.06 $\mu\text{g/g}$ f.w. in seeds, 3,817.68 $\mu\text{g/g}$ f.w. and 646.45 $\mu\text{g/g}$ f.w. in leaves, 3,283.97 $\mu\text{g/g}$ f.w. and 595.47 $\mu\text{g/g}$ f.w. in nodes and 3,014.72 $\mu\text{g/g}$ f.w. and 481.35 $\mu\text{g/g}$ f.w. in roots (Fig. 4a). Since, the optimum level of sennoside was available

Table 2 Effect of auxins supplemented to half strength MS medium on rooting of *in vitro* derived shoots of *C. angustifolia*. Experiment was repeated once bearing consistent results

Auxins (μM)	% Root induction*	Average no. of roots per shoot* (mean $\pm$ SE)	Average root length* (cm) (mean $\pm$ SE)
Control			
0	—	—	—
IBA			
0.5	45.85 ^b	0.87 $\pm$ 0.008 ^b	1.16 $\pm$ 0.017 ^a
1.0	62.53 ^d	2.07 $\pm$ 0.008 ^c	1.76 $\pm$ 0.006 ^c
5	70.86 ^c	3.55 $\pm$ 0.008 ⁱ	4.14 $\pm$ 0.009 ^j
10	91.64 ^g	5.14 $\pm$ 0.007 ^k	4.76 $\pm$ 0.007 ^k
NAA			
0.5	45.85 ^b	0.75 $\pm$ 0.010 ^a	2.33 $\pm$ 0.008 ^c
1.0	70.85 ^c	1.83 $\pm$ 0.007 ^d	3.08 $\pm$ 0.007 ^g
5	62.53 ^d	2.86 $\pm$ 0.006 ^g	3.47 $\pm$ 0.007 ⁱ
10	87.53 ^f	3.96 $\pm$ 0.008 ^j	3.06 $\pm$ 0.010 ^g
IAA			
0.5	37.53 ^a	0.75 $\pm$ 0.009 ^a	1.57 $\pm$ 0.017 ^b
1.0	54.17 ^c	1.54 $\pm$ 0.007 ^c	2.06 $\pm$ 0.006 ^d
5	70.85 ^c	2.33 $\pm$ 0.009 ^f	3.25 $\pm$ 0.010 ^h
10	70.86 ^c	3.29 $\pm$ 0.007 ^h	2.47 $\pm$ 0.007 ^f

*Mean values in a column followed by different letters are significantly different as determined by SPSS at $p=0.05$ according to DMRT

Table 3 List of RAPD primers, their sequences, number of scorable bands and size of amplified fragments generated in *C. angustifolia* donor plant and regenerants

Sl. no.	Primer	Sequence (5'–3')	No. of scorable bands	No. of monomorphic bands	No. of polymorphic bands	Size of bands (bp)
1	VAB-01	GTTTCGCTCC	3	3	0	300–700
2	VAB-04	GGACTGGAGT	4	4	0	200–600
3	VAB-05	TGCGCCCTTC	5	5	0	300–1,000
4	VAB-06	TGCTCTGCCC	4	3	1	300–900
5	VAB-10	CTGCTGGGAC	3	2	1	200–700
6	VAB-11	GTAGACCCGT	4	4	0	400–900
7	VAB-12	CCTTGACGCA	5	5	0	100–550
8	VAB-17	AGGGAACGAG	3	3	0	200–600
9	VAB-19	ACCCCCGAAG	3	3	0	100–400
10	VAB-20	GGACCCTTAC	2	2	0	200–500
Total			36	34	2	

in leaf, a comparison of sennoside A and B content was done between leaves of micropropagated plants of *C. angustifolia* and donor elite plant. HPLC chromatogram of the sennoside A and B content from leaves of elite mother stock and 11 *ex vitro* regenerated mature plants individually as well as of the pooled leaf samples showed consistency in their content. The average content of mature regenerants by randomly selecting

two micropropagated individuals for their sennoside A and B content was 3,822.72 $\mu\text{g/g}$ f.w. and 650.84 $\mu\text{g/g}$ f.w., respectively whereas, for the mother plant was 3,817.96 $\mu\text{g/g}$ f.w. and 646.77 $\mu\text{g/g}$ f.w., respectively (Fig. 4b–d). A further consistency in sennoside A and B content was revealed by evaluating pooled leaf samples of mature regenerants with the donor elite mother plant (Fig. 4d).

Fig. 2 Establishment of genetic fidelity among the mature regenerants of *C. angustifolia*. **a** & **b** RAPD profile of the primers VAB-05 (**a**) and VAB-10 (**b**) of the 11 regenerants and elite mother plant of *C. angustifolia*

(Lanes *M*: 100 bp DNA size Marker, *M.P.*— elite mother plant, *1* to *11*: micropropagated plants of *C. angustifolia*)

Fig. 3 HPLC chromatograms of standard compounds, sennoside A and B revealing peaks at retention time of ~34.7 (a) and ~30.7 min (b), respectively. c–f HPLC peaks of sennoside A and B of leaf (c), node (d), root (e) and seed (f)

Discussion

Development of protocol for direct *in vitro* shoot regeneration from field raised elite plant and their validation through fingerprinting is an urgent need toward clonal mass multiplication and for commercial/pharmaceutical application. Following the current protocol, it has been possible to directly regenerate shoots from nodal explants of field raised elite plant of *C. angustifolia*. Similarly, efficient regeneration protocol of an elite clone of *Cullen corylifolium* yielding maximum content of psoralen was carried out through selection of the elite psoralen yielding chemotype (Chetri et al. 2014). Among the three different cytokinins employed, viz. BA, Kn and 2iP, BA proved to be the best both in terms of percentage morphogenic cultures as well as average shoot number per explant. The efficiency of BA over other cytokinins could be due to its easy permeability, increased affinity for active cell uptake, less resistance to the enzyme cytokinin oxidase, or receptor abundance in its perception apparatus which interacts with the

coupling elements in the signal transduction chain (Burch and Stuchbury 1987). BA if added exogenously shortens the duration of S-phase of cell division through recruitment of latent origin of DNA replication both *in vitro* or *in vivo* (Francis and Sorell 2001). BA was also reported for induction of multiple shoots during *in vitro* culture of cotyledonary node, nodal explant (Agrawal and Sardar 2003), leaf explant (Agrawal and Sardar 2006), petiole explant (Siddique et al. 2010), root explant (Parveen and Shahzad 2011) and cotyledon explant (Agrawal and Sardar 2007; Parveen et al. 2012) of *C. angustifolia*. Significance of BA in inducing multiple shoots has been already reported in *Simmondsia chinensis* (Link) Schneider (Agrawal et al. 2002) and *Spilanthes* spp. (Razaq et al. 2013).

For rhizogenesis, IBA yielded the optimum response in terms of percentage root induction and average root number per shoot. NAA and IAA when tried alone induced callusing prior to rooting. As mentioned earlier (Zolman et al. 2000), IBA is the most widely used

Fig. 4 Establishment of biochemical fidelity in terms of sennoside A and B content amongst the mature regenerants of *C. angustifolia*. **a** Graphical representation of the estimation of sennoside A and B content through HPLC analysis from different plant parts of *C. angustifolia*. Values with different letters at the top of columns show significant difference according to DMRT at $p=0.05$. **b & c** HPLC peaks of sennoside A and B of leaf samples of elite mother genotype and pooled leaf samples from the mature regenerated plants of *C. angustifolia*, respectively. **d** Graphical representation of consistency in sennoside A and B content of the mature regenerated plants and elite mother plant of *C. angustifolia*. Values with same letters at the top of columns are not significantly different according to DMRT at $p=0.05$

auxin to stimulate the rooting process in shoots because of its high ability to promote root initiation, its weak toxicity and improved stability in comparison to NAA and IAA (Weisman et al. 1988). Several workers while working on different explants of *C. angustifolia* revealed the optimum rooting response on IBA

supplemented medium (Agrawal and Sardar 2006, 2007; Siddique et al. 2010; Parveen and Shahzad 2011; Parveen et al. 2012). It is also reported that the application of IBA under *in vitro* conditions may induce changes in enzyme activities (peroxidase and IAA oxidase) and in their effectors contents (phenolics) allowing

the establishment of the favourable endogenous hormone balance for root initiation in excised shoots (Qaddoury and Amssa 2004).

Another important feature of this investigation is the establishment of genetic fidelity amongst the micropropagated plants employing PCR based RAPD marker which has been done for the first time here. The banding profiles of micropropagated plants vis-a-vis that of mother plants shown by RAPD marker has clearly revealed almost complete uniformity and monomorphism thereby, indicating a high level of genetic similarity amongst the regenerants and the elite donor plant of *C. angustifolia*. The low level of genetic variation in DNA may be attributed to naturally occurring variation or due to the accumulation of mutation by various factors such as *in vitro* process and its duration, auxin to cytokinin ratio (hormonal balance), *in vitro* stress induced by added biochemicals, or other nutritional conditions (Razaq et al. 2013; Singh et al. 2013). The genetic variation induced by such genetic and epigenetic mechanism can be reflected in the banding profile developed by different marker system (Phillips et al. 1994). This further supports the need for testing micropropagated plantlets well before their actual planting in the field and confirming the reliability of the micropropagation protocol for large scale exploitation. In the present study, we adopted the use of PCR-based RAPD marker for establishment of genetic fidelity amongst the regenerants of *C. angustifolia*.

Yet another novel parameter mentioned here is the establishment of consistency in secondary metabolite (sennoside A and B) content among the regenerants and the mother plant. This has also been done for the first time in this plant. Similar to our work, consistency in content of bioactive molecules of *in vitro* derived mature plants with the mother stock was earlier reported in *Nothapodytes nimmoniana* (Dandin and Murthy 2012) and *Cullen corylifolium* (Chetri et al. 2014). The results presented here demonstrated a highly efficient and viable protocol for mass production of true to the type plants of *C. angustifolia*. Based on these results, it is emphasized that the protocol developed here, is efficient in terms of genetic fidelity and sennoside content and therefore, can be gainfully employed for mass propagation and for pharmaceutical industries.

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Conflict of interest The authors declare that they have no conflict of interest.

Author contributions statement This paper pertains to direct regeneration through mature *in vivo* nodal explants of *Cassia angustifolia* and establishment of genetic and biochemical uniformity amongst the regenerants employing RAPD and HPLC, respectively. All the authors have equal contribution for the research paper.

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