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ORIGINAL ARTICLE

High-frequency clonal propagation of *Curcuma angustifolia* ensuring genetic fidelity of micropropagated plants

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Abstract

A high-frequency clonal propagation protocol was developed for *Curcuma angustifolia* Roxb., a high valued traditional medicinal plant. Axillary bud explants of *C. angustifolia* were explanted on Murashige and Skoog (MS) medium fortified with 4.4–22.2 µM 6-benzyladenine (BA), 2.9–5.7 µM indole-3-acetic acid (IAA), 2.3–23.2 µM kinetin (Kin), 2.7–5.4 µM naphthalene acetic acid (NAA) and 67.8-271.5 µM adenine sulphate (Ads) in different combinations. The maximum number of shoots per explants (14.1 ± 0.55) and roots per shoot (7.6 ± 0.47) was achieved on media containing 13.3 µM BA, 5.7 µM IAA and 135.7 µM Ads. Stability in phytomedicinal yield potential of micropropagated plants was assessed through GC–MS and HPTLC. Gas chromatogram of essential oil of conventional and micropropagated plants of *C. angustifolia* had similar essential oil profile. HPTLC analysis of rhizome extracts of in vitro and field grown plants revealed no significant differences in the fingerprint pattern and in curcumin content. Genetic integrity of in vitro and field grown derived plants were evaluated with inter-simple sequence repeat (ISSR) primers and flow cytometry using *Glycine max* as an internal standard. A total of 1260 well resolved bands were generated by 12 ISSR primers showing monomorphic banding patterns across all plants analyzed. The mean 2C DNA content of conventionally and micropropagated plant was estimated to be 2.26 pg and 2.31 pg, respectively. As no somaclonal variations were detected in tissue culture plantlets, the present micropropagation protocol could be applied for in vitro conservation and large-scale production of *C. angustifolia*.

Keywords Micropropagation · *Curcuma angustifolia* · Genetic stability · GC–MS · Essential oil · HPTLC

Introduction

Curcuma angustifolia Roxb. (East Indian Arrowroot) belonging to family Zingiberaceae is a rhizomatous perennial flowering herb found throughout Eastern India, Central and Southern India (Sharma 2012). Traditionally, East Indian Arrowroot is used as a herbal remedy for healing various ailments such as kidney disorders, thirst, fever, flattening the body, leucorrhoea, headache and remedy for joint pains (Srivastava et al. 2006; Hemadri and Rao 1984). The starch of *C. angustifolia* used as Tugaksheeree is an important ingredient extensively used in the preparation of Ayurvedic medicines (Rajashekhara and Sharma 2010). The essential oil extracted from this plant possess numerous pharmacological activities like antifungal, antibacterial and antioxidant activities (Jena et al. 2017).

The conventional method of vegetative propagation of *C. angustifolia* is not satisfactory because of slow multiplication rate (Shukla et al. 2007) and high incidence of rhizomes to pathogenic diseases in field and storage conditions. Besides this, overexploitation of the plant from natural sources has resulted in unavailability of requisite quality planting material of *C. angustifolia*. Thus it was felt necessary to develop a high-frequency plant regeneration system for commercial production of *C. angustifolia* through tissue culture. Plant tissue culture is an efficient tool for the rapid multiplication and conservation of different plant species in a controlled environment for large scale production on a commercial scale (Sliwinska and Thiem 2007; Singh et al.

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2011; Al Khateeb et al. 2012). Several thousands of plantlets can be multiplied from a single explant within a limited time duration as well as limited space under the controlled environment, without seasonal changes and climate all over the year (Akin-Idowu et al. 2009; Amoo et al. 2012). Efficient micropropagation protocols have been reported in several *Curcuma* species, such as *C. longa* (De Souza Ferrari et al. 2016), *C. amada* (Barthakur and Bordoloi 1992), *C. aromatica* (Sharmin et al. 2013), *C. mangga* (Raihana et al. 2011) and *C. zedoaria* (Anisuzzaman et al. 2008). However in *C. angustifolia*, existing tissue culture report (Shukla et al. 2007) necessitated further work on the establishment of high-frequency plant regeneration system for the commercial purpose.

For the commercial application of micropropagation techniques, it is a requisite to maintain genetic fidelity among in vitro regenerants, because a change in the in vitro culture can result in emergence of somaclonal variations (Larkin and Scowcroft 1981; Nayak et al. 2011). Therefore it is necessary to monitor the genetic fidelity of the in vitro regenerants after acclimatization to ex vitro conditions. DNA based markers are an efficient tool to check the genetic fidelity in micropropagated plants as they are unaffected by environmental conditions (Peredo et al. 2009). Polymerase chain reaction (PCR) based techniques like inter-simple sequence repeat (ISSR), simple sequence repeat (SSR), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), retrotransposon microsatellite amplified DNA polymorphism (REMAP) are being widely used to confirm genetic fidelity (Bairu et al. 2011; Aversano et al. 2011; Nayak et al. 2011). Among these DNA markers, ISSR markers are highly polymorphic, simpler, quicker and powerful methods for the evaluation of genetic integrity of tissue culture derived plants and overcomes most of the limitation of the other techniques (Viehmannova et al. 2016; Raji et al. 2017).

Further, now-a-days, flow cytometry analysis are used to detect genome size and ploidy changes among micropropagated plants (Bairu et al. 2011; Shilpha et al. 2014; Choudhury et al. 2014; Raji et al. 2017). Through, flow cytometry, it is possible to analyze large numbers of nuclei in a shorter time period (Viehmannova et al. 2016). It is difficult to genetically control the traits that are associated with biochemical characters (Nayak et al. 2011; Singh et al. 2011). Therefore, it is crucial to carry out stability assessment of phytomedicinal yield potential of in vitro regenerants of commercial important medicinal plants.

A previous report on in vitro propagation of *C. angustifolia* by Shukla et al. (2007) exclude any detailed analysis regarding the genetic integrity of micropropagated plants and assessment of their phytomedicinal yield potentials which is the most crucial aspect for commercial application of any micropropagated plant. For stability assessment of regenerants, a combination of several techniques should be used rather than using only one (Peredo et al. 2009; Nayak et al. 2011). Therefore, the present work was aimed to develop high-frequency plant regeneration protocol through axillary bud formation. Further evaluation of genetic stability of tissue culture derived plants of *C. angustifolia* was carried out by ISSR and flow cytometry analysis. GC–MS and HPTLC analysis was used to analyze the essential oil and curcumin content. The reported tissue culture technique would be of enough significance for large scale production of *C. angustifolia* for a stable supply of drugs of uniform quality for commercial use.

Materials and methods

Collection of plant materials

Healthy rhizomes of *C. angustifolia* collected from R-Udaygiri, Gajapati district, Odisha were maintained in the greenhouse of Centre for Biotechnology, Siksha 'O' Anusandhan (Deemed to be University, Odisha, India) until initiation of sprouting. Immature dormant rhizome axillary buds were excised with sharp blade and were washed properly with distilled water to remove dirt from the surface and then soaked in liquid detergent (Extran, Merck, Mumbai, India) for 3–5 min and subsequently rinsed with distilled water, which were used as the explants. Surface sterilization of explants were performed with 0.1% (w/v) Mercury chloride ($HgCl_2$) solution for 8–10 min and followed by washing with autoclaved distilled water several times before inoculation.

Establishment of plant tissue culture

Surface sterilized explants were explanted aseptically on autoclaved basal medium of Murashige and Skoog (MS) (1962) containing 4.4–22.2 µM 6-benzyladenine (BA), 2.9–5.7 µM indole-3-acetic acid (IAA), 2.3–23.2 µM kinetin (Kin), 2.7–5.4 µM naphthalene acetic acid (NAA) and 67.8-271.5 µM adenine sulphate (Ads) in different combinations. The pH of all the media containing the varying combination of hormones was adjusted to 5.8. The media containing 0.8% (w/v) agar was then autoclaved at 121 °C and for 20 min. After inoculation, all the cultures were maintained at 25 ± 1 °C under a 16:8 h light/dark cycles provided by white fluorescent light with 50 µmol m^2/s light intensity. After 8 weeks of culture in vitro, number of shoots per explants and number of roots per shoot were recorded. For in vitro multiplication, twenty explants were raised for each experiment and experiments were repeated three times. MS basal medium devoid of any plant growth regulators used as control.

Acclimatization

The in vitro regenerants having well-developed roots as well as shoots were taken out from the culture tube and washed properly. After washing, plantlets were further transplanted to pots containing soil, cow dung, and sand mixture in 1:1:1 ratio for acclimatization in the growth chamber at a high relative humidity (80–90%) and low temperature (24–25 °C) with 12 h photoperiod for 15 days. Finally acclimatized plantlets were later transferred to the field and grown to maturity. 2 year of field grown tissue culture derived and conventionally propagated plantlets of *C. angustifolia* were compared for different biochemical parameters.

Extraction of essential oil and GC–MS analysis

For GC–MS analysis of essential oil, rhizome and leaf of following plant material was selected: in vitro derived plants obtained from axillary buds and cultured on MS medium in the presence of 13.3 µM BA, 5.7 µM IAA and 135.7 µM Ads and maintaining it for 1 year by subculturing it every 8 weeks and then transferring to field for 2 years and conventionally propagated plants grown in field for 2 years was used for comparison. Essential oil was extracted using hydrodistillation according to the method of Guenther (1972) and the extracted essential oil was dehydrated over anhydrous Na_2SO_4. The analysis of essential oils was achieved by the Clarus 580 Gas Chromatograph (Perkin-Elmer, USA) coupled with a SQ-8 MS detector. Compounds separation was performed on Elite-5 MS capillary column (5% phenyl, 95% dimethyl polysiloxane, Perkin Elmer, USA) having 30 m length × 0.25 mm I.D. × 0.25 µm film thickness. Oven temperature was programmed at 60 °C then increased to 220 °C at 3 °C/min, and then finally kept at 220 °C for 7 min. The injector temperature was 250 °C. Helium was used as carrier gas with flow rate 1 mL/min. Transfer interface and source temperature programming was kept at 250 °C. The electron ionization source was used at 70 eV.

Gas chromatography (GC) analysis was done by Clarus 580 (Perkin Elmer, USA) gas chromatograph coupled with a Flame ionization detector (FID). Injector and detector (FID) temperatures were set at 250 °C. All programming temperatures were similar to that of the GC–MS. Further, the area percentages of the detected compounds were computed from the GC-FID peak areas. Identification of various constituents was based on by comparing the mass Spectra (MS) data obtained from each constituent with NIST library. Further identification was supported by calculating relative retention indices (RRI) using homologous n-alkane (C_8–C_{20}) series as external references and comparing its values with the published literature (Adams 2007). RI was determined according to the equation of Van den Dool and Kratz (1963).

Sample preparation and HPTLC analysis

In vitro regenerants obtained from axillary buds cultured on MS medium supplemented with 13.3 µM BA, 5.7 µM IAA and 135.7 µM Ads and maintaining it for 1 year by subculturing it every 8 weeks and then transferring to field for 2 years. For comparison conventionally propagated plants grown in field for 2 years was used. The rhizomes of both the plants were powdered and were subjected to extraction in methanol in soxhlet apparatus for 6 h. Then all the individual extract were combined and were concentrated under vacuum using Rotary evaporator. The extract was then stored in air-tight container at 4 °C for further analysis. 1 mg of curcumin (analytical grade, purity > 98%, Sigma Aldrich, USA) was diluted in 10 ml HPLC-grade methanol. Similarly, 10 mg of extract (micropropagated and conventionally propagated plant of *C. angustifolia*) was reconstituted in 10 ml of methanol for HPTLC analysis. Spots of extracts and standards were applied as 6 mm wide bands positioned 10 mm from the bottom and 10 mm from the side of the plate, using Linomat V applicator (Camag, Muttenz, Switzerland). The sample was applied into the aluminium plates, precoated with silica gel 60 F_{254} (20 × 20 cm, 0.2 mm thick, Merck Ltd, Mumbai) at an application rate of 200 nl/s from 100 µl syringe. Standard solution volumes of 2–10 µl of curcumin (0.1 mg/ml) were applied onto the plate corresponding to a concentration of 200–1000 ng for the preparation of a calibration curve. The calibration curve showed linearity over the evaluated ranges with linear equation of $y = 96\,x + 34.69$ and correlation coefficient of determination ($R^2 = 0.997$). Separation was carried out in an ascending mode in a twin trough chamber pre-saturated for 15 min with mobile phase, *n*-hexane: ethyl acetate (85:15 v/v; 20 ml). The plate was dried in air under room temperature and scanned in absorbance/reflectance mode under TLC scanner 4 at 366 nm. Peak area data were recorded using Win CATS software. Quantification of curcumin was carried out on the basis of regression equation of the standard curve of curcumin.

ISSR analysis

Genomic DNA was isolated from fresh leaves of 20 plantlets derived from axillary buds cultured on MS medium in the presence 13.3 µM BA, 5.7 µM IAA and 135.7 µM Ads and maintaining it for 1 year by subculturing it every 8 weeks and then transferring to field for 2 years. The leaves of conventionally propagated plants were used for comparison. Total DNA was extracted following the protocol of Doyle and Doyle (1987) cetyltrimethylammonium bromide (CTAB) method with minor modification. Purification of DNA was performed with RNase A (60 µg/µl of crude DNA solution) followed by washing three times with chloroform: isoamyl alcohol (24:1). The purified DNA was quantified

using a spectrophotometer by taking absorbance at 260/280 ratio and its quality checked by using 0.8% agarose gel. Then the final concentration of each sample was adjusted to 25 ng/µl for PCR with Tris–EDTA buffer (pH 8.0) and kept at − 20 °C until analysis. Amplifications were carried out in a reaction mixture of 25 µl containing 25 ng of genomic DNA as a template, 10X PCR buffer mixed with 15 mM $MgCl_2$ (Bangalore Genei), 200 µM dNTPs (Bangalore Genei), 3 U/µl of Taq DNA polymerase (Bangalore Genei) and 5 pM of each primer. The amplification of the reaction was carried out in a thermal cycler (Applied Biosystems Veriti 96 Well Thermal Cycler) under the following program: initial denaturation for 5 min at 94 °C, followed by 40 cycles at 94 °C for 1 min (denaturation) then anneling temperature for 1 min and then 72 °C for 2 min (extension), followed by final extension at 72 °C for 7 min. The PCR products were separated using 1.5% agarose gel stained with ethidium bromide (0.5 µg/ml). The allele sizes were evaluated by comparing 100 bp plus DNA ladder (Thermo scientific) and documented using Bio-Rad gel documentation system.

Flow cytometry analysis

Nuclear DNA content was analyzed using BD FACS Calibur flow cytometer (Becton–Dickinson Immunocytometry System, San Jose, CA, USA). The genetic integrity was analyzed by comparing 2C DNA content of in vitro grown regenerants and conventionally propagated plant of *C. angustifolia. Glycine max* (2C DNA = 2.50 pg, Dolezel et al. 1994) was used as internal reference standard. Sample analysis was carried out following the protocol of Choudhury et al. (2014) using Otto I buffer, 50 µM propidium iodide and 100 µM RNAse. For each sample, 10,000 nuclei were measured using BD Cell Quest Pro. Analysis of measurement was carried out in FCS Express 4 software. Randomly chosen regenerants obtained from the most effective combination for in vitro multiplication was assessed to check the 2C DNA content. The nuclear DNA content of *C. angustifolia* was estimated by the following equation:

$$\text{2C DNA content of } C.\ angustifolia = \text{2C DNA content of internal standard} \times \frac{\text{Mean of G0/G1 peak of } C.\ angustifolia}{\text{Mean of G0/G1 peak of } Glycine\ max}$$

Statistical analysis

The data were subjected to Analysis of variance (ANOVA) followed by Tukey's HSD test at $p < 0.05$. All the analysis was carried out in Minitab 17 statistical software (Minitab Inc, PA, USA).

Results

In vitro establishment and multiplication of plants

A morphologically and phenotypically healthy plant was selected as mother plant for culture establishment (Fig. 1A). The dormant axillary buds of field grown *C. angustifolia* were taken as explants source (Fig. 1B). Explants were inoculated vertically into MS medium fortified with different concentrations of auxins and cytokinins. MS medium supplemented with cytokinins like BA and Kin brought changes in sprouted bud explants and bud induction was visible within 30 days. BA was found to be the better as compared to Kin in terms of shoot induction and multiplication. The multiplications of axillary buds were increased, when the concentrations of BA were increased from 2.9 to 13.3 µM in the culture medium (Table 1). However, BA concentration beyond 13.3 µM did not manifest any increase in the number of shoot formation. Among all the media combinations, the maximum response of shoot initiation (90%) was observed with two media concentrations, i.e., the media containing 13.3 µM BA and 2.9 µM IAA as well as in the media with 13.3 µM BA, 2.9 µM IAA and 135.7 µM Ads. Among all tried combinations, Kin at 23.2 µM was found to be least effective. The percentage of aseptic axillary buds obtained from rhizome after the sterilization process varied from 45 to 95% for different phytohormones combination and is mentioned as % of shoot initiation in Table 1. Approximately 14 plantlets were produced from a single explants and the shoot multiplication time was 2 months. Therefore approximately 38,416 plantlets can be produced from single explants in 6 month where as multiplication rate through traditional method can be maximum 7 plants per annum. Almost 95% of rhizome buds sprouted on media supplemented with 13.3 µM BA, 2.9 µM IAA and 135.7 µM Ads giving rise to an average of 14.1 ± 0.55 shoots and 7.6 ± 0.47 of roots within 4 weeks (Fig. 1C). For rooting the same media combination was tested as that used for shoot induction. In vitro culture was maintained by sub culturing regularly at 2 month interval. After maintaining, in vitro tissue culture in the culture medium for 1 year, they were transferred to pots containing soil, cow dung, and sand mixture in 1:1:1 ratio for hardening (Fig. 1D). Plants were acclimatized in the greenhouse for 30 days and about 95% of plants survived and had normal growth after transferring them to the field during the planting season (Fig. 1E).

Assessment of phytomedicinal yield potential of regenerants

The phytomedicinal yield potential of regenerants were assessed through analysis of essential oil and curcumin

Fig. 1 *Curcuma angustifolia* (**A**) control plant (**B**) explant at the time of culture (**C**) multiple shoot induction within 8 weeks supplemented with 13.3 µM BA, 5.7 µM IAA and 135.7 µM Ads (**D**) plants transferred to ex vitro conditions (**E**) acclimatized plantlets after 2 years in field condition

content. The leaf oil yield of conventionally propagated and micropropagated plant of *C. angustifolia* were 0.30 and 0.32% (v/w), respectively, whereas, oil yield of rhizome of conventionally propagated and micropropagated plant of *C. angustifolia* were 0.40 and 0.45% (v/w), respectively. The essential oils extracted from rhizome and leaf of in vitro propagated and field grown plants of *C. angustifolia* were subjected to GC–MS analysis (Tables 2, 3). The rhizome essential oil in vitro propagated and field grown plants of *C. angustifolia* revealed the presence of 35 constituents, representing 94.21 and 92.31% of the total peak area, respectively (Fig. 2). Similarly, leaf essential oil showed the presence of 32 components, representing 94.35 and 92.57% of total volatile oil content in in vitro propagated and field grown plants, respectively (Fig. 3). Major constituents like curzerenone, camphor, germacrone, xanthorrhizol, β-eudesmol were common to both in vitro derived and field grown plants rhizome oil. Similarly, leaf oil revealed the presence of predominant components such as curzerenone, 14-hydroxy-δ-cadinene, γ-eudesmol acetate, (Z)-α-Santalol acetate, germacrone that were common to both in vitro derived and source plants. Rhizome and leaf essential oil of both conventionally propagated and micropropagated plant of *C. angustifolia* were dominated by curzerenone that comprises of 72.55 and 33.23% of peak area, respectively.

For HPTLC analysis, different trials were made using different solvents in different proportions so as to acquire compact and precise bands. HPTLC analysis revealed that chromatogram of the conventionally propagated plant of *C. angustifolia* had similar fingerprint pattern to that of micropropagated plants (Fig. 4). The chromatograms obtained from field grown and in vitro derived plants of extract of *C. angustifolia* exhibited peaks corresponding to R_f values of 0.09, 0.14, 0.17, 0.25, 0.37, 0.44, 0.50, 0.65, 0.89 at 254 nm. Mobile phase containing *n*-hexane:ethyl acetate (85:15) gave a compact peak at R_f 0.17 for curcumin in both conventionally propagated and micropropagated methanol extract (Fig. 4A, B) and reference curcumin (Fig. 4C). The presence of curcumin in extract source plant and micropropagated plant was further confirmed by super impose overlay UV spectra of the corresponding peak in standard and extract (Fig. 5). The percentage of curcumin content determined using calibration curve was found to be $0.11 \pm 0.01\%$ and $0.13 \pm 0.01\%$, in conventionally propagated and micropropagated plants of *C. angustifolia*, respectively.

Table 1 Effect of various plant growth regulators on in vitro shoot initiation and multiplication of *C. angustifolia*

MS media with growth regulators (μM)					% of shoot initiation	No of shoots per explants	No of roots per shoot
BA	Kin	IAA	NAA	Ads			
0.0	0.0	0.0	0.0	0.0	0	0.0 ± 0.00^{u}	0 ± 0.00^{n}
4.4	–	–	–	–	65	4.3 ± 0.10^{pq}	2.6 ± 0.13^{lm}
13.3	–	–	–	–	75	7.6 ± 0.32^{efg}	4.1 ± 0.22^{fgh}
22.2	–	–	–	–	80	5.5 ± 0.11^{klmno}	2.4 ± 0.06^{m}
–	4.6	–	–	–	65	2.5 ± 0.13st	3.7 ± 0.11^{ghi}
–	13.9	–	–	–	75	4.6 ± 0.23^{opq}	2.9 ± 0.15^{klm}
–	23.2	–	–	–	45	1.5 ± 0.06^{t}	3.1 ± 0.08^{ijkl}
4.4	–	2.9	–	–	50	3.1 ± 0.25^{rs}	4.5 ± 0.17^{def}
4.4	–	5.7	–	–	55	5.3 ± 0.43^{mnop}	3.0 ± 0.12^{jklm}
8.9	–	2.9	–	–	50	4.2 ± 0.31^{q}	4.3 ± 0.18^{efg}
8.9	–	5.7	–	–	60	6.5 ± 0.34^{hijk}	4.7 ± 0.09^{def}
13.3	–	2.9	–	–	65	4.1 ± 0.17^{qr}	3.3 ± 0.07^{ijk}
13.3	–	5.7	–	–	90	11.6 ± 0.44^{b}	6.4 ± 0.16^{b}
8.9	–	–	2.7	–	70	6.6 ± 0.52^{ghij}	4.5 ± 0.21^{def}
8.9	–	–	5.4	–	55	5.5 ± 0.37^{klmno}	4.7 ± 0.16^{def}
13.3		–	2.7	–	75	7.1 ± 0.42^{fghi}	4.2 ± 0.29^{efgh}
13.3	–	–	5.4	–	50	5.4 ± 0.22^{lmno}	2.8 ± 0.11^{klm}
8.9	–	2.9	2.7	–	85	7.5 ± 0.42^{efgh}	4.8 ± 0.27^{de}
13.3	–	5.7	2.7	–	80	8.3 ± 0.55^{de}	4.4 ± 0.38^{ef}
8.9	2.3	–	2.7	–	55	5.1 ± 0.22^{nopq}	3.3 ± 0.12^{ijk}
8.9	4.6	–	5.4	–	75	7.6 ± 0.38^{efg}	4.2 ± 0.21^{efgh}
13.3	2.3	–	–	–	70	6.4 ± 0.27^{ijkl}	4.3 ± 0.19^{efg}
13.3	4.6	–	–	–	80	8.1 ± 0.42^{ef}	4.5 ± 0.34^{def}
–	13.9	–	2.7	–	75	5.7 ± 0.18^{jklmn}	4.8 ± 0.27^{de}
–	13.9	–	5.4	–	70	4.6 ± 0.13^{opq}	4.1 ± 0.18^{fgh}
–	13.9	2.9	–	–	55	6.2 ± 0.31^{ijklm}	3.3 ± 0.16^{ijk}
–	13.9	5.7	–	–	50	5.4 ± 0.16^{lmno}	3.6 ± 0.15^{hij}
–	13.9	2.9	–	135.7	50	5.4 ± 0.08^{lmno}	2.9 ± 0.12^{klm}
–	13.9	2.9	–	271.5	55	4.7 ± 0.11^{pqr}	3.1 ± 0.14^{ijkl}
–	13.9	5.7	–	135.7	60	4.9 ± 0.13^{pqrs}	2.6 ± 0.07^{lm}
–	13.9	5.7	–	271.5	45	3.8 ± 0.05^{rs}	2.8 ± 0.11^{klm}
13.3	–	2.9	–	67.8	70	9.9 ± 0.49^{c}	6.0 ± 0.29^{bc}
13.3	–	2.9	–	135.7	75	11.9 ± 0.31^{b}	6.8 ± 0.23^{b}
13.3	–	2.9	–	203.6	70	10.6 ± 0.31^{c}	6.2 ± 0.18^{b}
13.3	–	2.9	–	271.5	65	10.3 ± 0.39^{c}	5.7 ± 0.17^{c}
13.3	–	5.7	–	67.8	65	12.4 ± 0.62^{a}	6.5 ± 0.28^{b}
13.3	–	5.7	–	135.7	95	14.1 ± 0.55^{a}	7.6 ± 0.47^{a}
13.3	–	5.7	–	203.6	85	11.3 ± 0.25^{b}	6.9 ± 0.36^{b}
13.3	–	5.7	–	271.5	85	9.2 ± 0.27^{d}	5.1 ± 0.19^{cd}

The values represent the means ± SD of three independent experiments. Twenty explants were raised for each experiment and experiment were repeated thrice. Mean having different letter in a column were significantly according to Tukey's HSD test at $P < 0.05$

BA 6 benzyladenine, *NAA* α-naphthaleneacetic acid, *IAA* indole-3-acetic acid, *Kin* kinetin, *Ads* adenine sulphate

Genetic stability of regenerants using ISSR markers and flow cytometry

ISSR marker based profiling of micropropagated and field grown plants of *C. angustifolia* were carried out to confirm whether the in vitro regenerants were genetically similar or not with the field grown plants. A total of twenty randomly selected in vitro derived plants and a mother plant were taken. Out of 27 ISSR primers tested, only 12 primers gave 60 well resolved and reproducible bands ranging from

Table 2 Chemical constituents identified in rhizome oil of both micropropagated and conventionally propagated *C. angustifolia*

No.	Compound	RI[a]	RI[b]	Peak area %	
				Conventionally propagated	Micropropagated
1	α-Thujene	929	924	0.11 ± 0.01	0.19 ± 0.01
2	α-Pinene	932	932	0.11 ± 0.01	0.14 ± 0.01
3	α-Fenchene	945	945	0.21 ± 0.01	0.28 ± 0.02
4	Camphene	949	946	0.36 ± 0.02	0.38 ± 0.02
5	β-Pinene	977	974	0.12 ± 0.01	0.15 ± 0.01
6	1,8-Cineole	1031	1026	0.15 ± 0.01	0.18 ± 0.01
7	(*Z*)-β-Ocimene	1029	1032	0.65 ± 0.11	0.72 ± 0.12
8	Linalool	1097	1095	0.45 ± 0.03	0.54 ± 0.02
9	*Cis*-Thujone	1099	1101	0.36 ± 0.02	0.47 ± 0.01
10	Camphor	1145	1141	3.26 ± 0.16	3.31 ± 0.13
11	Myrtenol	1193	1194	0.15 ± 0.01	0.16 ± 0.01
12	β-Elemene	1387	1389	0.45 ± 0.04	0.52 ± 0.03
13	(E)-Caryophyllene	1416	1417	0.46 ± 0.03	0.51 ± 0.01
14	ar-curcumene	1477	1479	0.27 ± 0.03	0.29 ± 0.01
15	Germacrene D	1484	1484	0.55 ± 0.04	0.61 ± 0.02
16	β-Selinene	1489	1489	0.96 ± 0.11	1.03 ± 0.07
17	Curzerene	1491	1495	0.55 ± 0.06	0.58 ± 0.04
18	γ-Cadinene	1514	1513	0.15 ± 0.01	0.27 ± 0.01
19	Caryophyllene oxide	1584	1584	0.12 ± 0.01	0.17 ± 0.01
20	Curzerenone	1603	1605	72.55 ± 0.95	72.62 ± 0.81
21	Humulene epoxideII	1618	1608	0.26 ± 0.01	0.33 ± 0.01
22	10-Epi-γ-eudesmol	1620	1622	0.12 ± 0.01	0.14 ± 0.01
23	γ-Eudesmol	1627	1630	0.11 ± 0.01	0.12 ± 0.01
24	epi-α-Cadinol	1638	1638	0.36 ± 0.02	0.39 ± 0.01
25	epi-α-Murrolol	1642	1640	0.15 ± 0.01	0.17 ± 0.01
26	β-Eudesmol	1650	1649	1.41 ± 0.13	1.5 ± 0.09
27	α-Cadinol	1653	1652	0.81 ± 0.08	0.95 ± 0.03
28	ar-Turmerone	1664	1668	0.14 ± 0.01	0.17 ± 0.01
29	epi-β-Bisabolol	1666	1670	0.12 ± 0.01	0.18 ± 0.02
30	Germacrone	1690	1693	3.25 ± 0.17	3.31 ± 0.13
31	Farnesol	1696	1698	0.10 ± 0.02	0.17 ± 0.01
32	Xanthorrhizol	1742	1751	2.83 ± 0.15	2.91 ± 0.13
33	(Z)-α-Santalol acetate	1775	1777	0.45 ± 0.03	0.49 ± 0.02
34	γ-Eudesmol acetate	1786	1783	0.11 ± 0.01	0.12 ± 0.01
35	β-Eudesmol acetate	1792	1792	0.12 ± 0.02	0.14 ± 0.01

Data are represented as mean ± SD (n = 3)

[a]Relative retention indices calculated against homologous *n*-alkane series (C_8–C_{20}) on the Elite-5 MS column

[b]Relative retention indices from literature (Adams 2007); Rhizome essential oils are derived from in vitro grown plants of *C. angustifolia* on MS medium with 13.3 µM BA, 5.7 µM IAA and 135.7 µM Ads

350 to 2300 bp in size. A total of 1260 well resolved bands were generated by ISSR analysis were showing uniformity in banding patterns across 20 tissue culture derived plants and mother plant tested (Fig. 6). The amplicon size of each primer is given in Table 4. The no. of bands for each primer varied from 3 to 8 with an average of 5 bands per ISSR primers. The highest band of (8) was observed against ISSR 20 and the lowest band of (3) against primer ISSR 12. Monomorphic patterns were observed among micropropagated and mother plant analyzed, showing no genetic variations among them (Fig. 6A, B).

Further genetic fidelity of the micropropagated plants with that of conventionally propagated plants of *C. angustifolia* was assessed by measuring their mean 2C DNA

Table 3 Chemical constituents identified in leaf oil of both micropropagated and conventionally propagated *C. angustifolia*

No.	Compound	RI[a]	RI[b]	Peak area %	
				Conventionally propagated	Micropropagated
1	α-Pinene	932	932	0.23 ± 0.02	0.27 ± 0.01
2	Camphene	949	946	0.49 ± 0.03	0.51 ± 0.01
3	β-Pinene	977	974	0.64 ± 0.02	0.73 ± 0.01
4	Limonene	1027	1024	0.16 ± 0.01	0.18 ± 0.01
5	1,8-Cineole	1031	1026	2.31 ± 0.15	2.37 ± 0.14
6	Cis-Thujone	1099	1101	0.16 ± 0.01	0.2 ± 0.01
7	Camphor	1145	1141	1.37 ± 0.09	1.39 ± 0.07
8	Camphene hydrate	1161	1145	0.33 ± 0.02	0.38 ± 0.01
9	Myrtenol	1193	1194	0.17 ± 0.01	0.19 ± 0.01
10	δ-Elemene	1333	1335	0.69 ± 0.03	0.72 ± 0.01
11	β-Elemene	1387	1389	2.77 ± 0.14	2.79 ± 0.11
12	(E)-caryophyllene	1416	1417	1.99 ± 0.19	2.05 ± 0.15
13	β-Gurjunene	1426	1431	0.12 ± 0.01	0.19 ± 0.01
14	α-Humulene	1451	1452	0.13 ± 0.01	0.21 ± 0.01
15	ar-curcumene	1477	1479	2.66 ± 0.22	2.73 ± 0.13
16	Germacrene D	1484	1484	0.20 ± 0.02	0.29 ± 0.01
17	Curzerene	1491	1495	2.45 ± 0.16	2.49 ± 0.11
18	β-Bisabolene	1503	1505	0.61 ± 0.11	0.71 ± 0.07
19	γ-Cadinene	1514	1513	0.49 ± 0.04	0.58 ± 0.01
20	Caryophyllene oxide	1584	1584	0.11 ± 0.05	0.17 ± 0.02
21	Curzerenone	1603	1605	33.23 ± 0.76	33.29 ± 0.71
22	Humulene epoxideII	1618	1608	0.25 ± 0.01	0.31 ± 0.01
23	γ-Eudesmol	1627	1630	0.21 ± 0.02	0.29 ± 0.01
24	epi-α-cadinol	1638	1638	0.50 ± 0.02	0.59 ± 0.02
25	epi-α-murrolol	1642	1640	0.14 ± 0.01	0.18 ± 0.01
26	β-Eudesmol	1650	1649	0.74 ± 0.03	0.77 ± 0.02
27	ar-Turmerone	1664	1668	0.15 ± 0.01	0.21 ± 0.01
28	Germacrone	1690	1693	3.68 ± 0.09	3.71 ± 0.06
29	Xanthorrhizol	1742	1751	5.14 ± 0.18	5.21 ± 0.12
30	(Z)-α-santalol acetate	1775	1777	4.29 ± 0.17	4.32 ± 0.13
31	γ-Eudesmol acetate	1786	1783	7.34 ± 0.27	7.44 ± 0.11
32	14-Hydroxy-α-cadinene	1800	1803	18.84 ± 0.75	18.88 ± 0.53

Data are represented as Mean ± SD (n = 3)

[a]Relative retention indices calculated against homologous *n*-alkane series (C_8–C_{20}) on the Elite-5 MS column

[b]Relative retention indices from literature (Adams 2007); Leaf essential oils are derived from in vitro plants of *C. angustifolia* on MS medium with 13.3 µM BA, 5.7 µM IAA and 135.7 µM Ads

content. Nuclear suspension of small pieces of young leaves from the conventionally propagated plant and plants regenerated from in vitro auxiliary bud was employed for this technique, and it exhibited clearly resolute linear histograms (Fig. 7). The linear histograms of relative nuclear content illustrated two peaks: the first showing nuclei in the G0/G1 phase of the cell cycle of *Glycine max* cv. Polanka and the second showing nuclei of the conventionally propagated *C. angustifolia* plants in the G0/G1 phase (Fig. 7A) and micropropagated plants in the G0/G1 phase (Fig. 7B). The mean 2C DNA content of micropropagated and conventionally propagated plant was estimated to be 2.31 pg and 2.26 pg, respectively, confirming similarity in genome size.

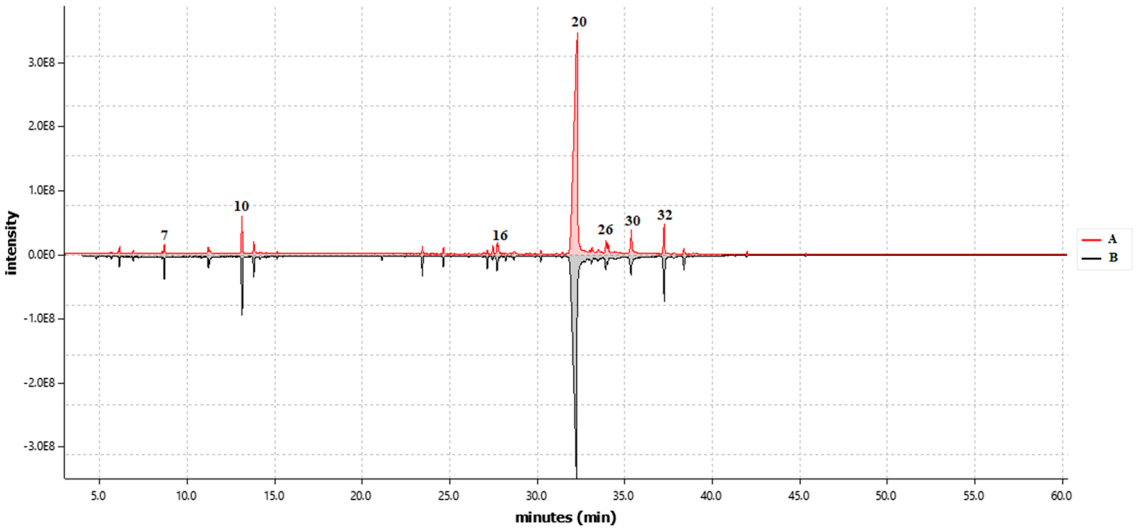

Fig. 2 GC–MS chromatogram of rhizome oil of *C. angustifolia* (**A**) conventionally propagated and (**B**) micropropagated plant as mirror image; the numbers above the peaks correspond to the compound listed in Table 2

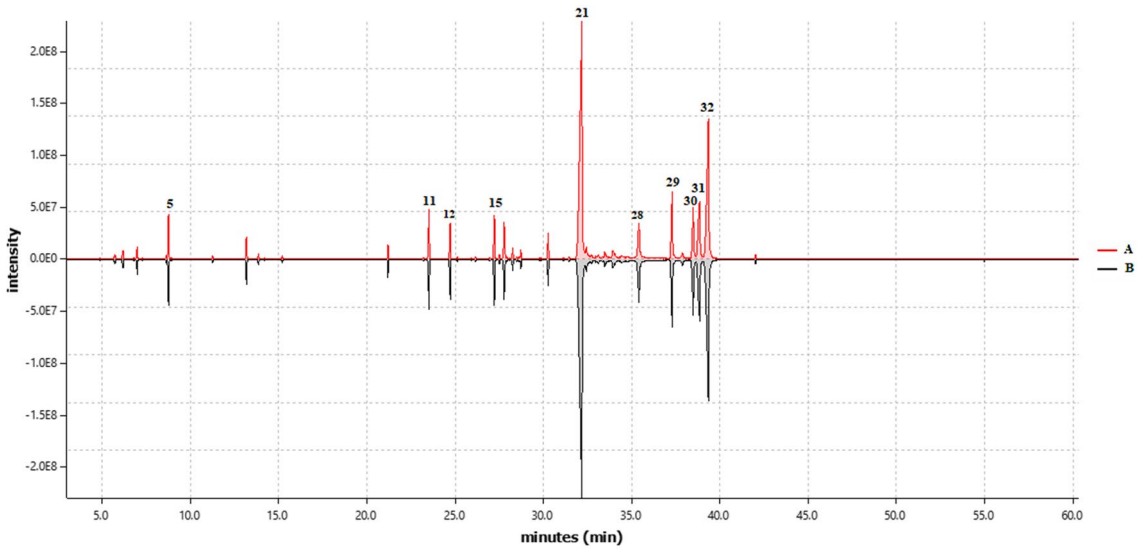

Fig. 3 GC–MS chromatogram of leaf oil of *C. angustifolia* (**A**) conventionally propagated and (**B**) micropropagated plants as mirror image; the numbers above the peaks correspond to the compound listed in Table 3

Discussion

Standardization of culture medium is an important factor for optimum growth response of the explants. BA, synthetic cytokinin triggers breaking the bud, induce auxiliary shoot formation and influence in vitro multiplication (Sadeghi et al. 2015). Earlier studies on *C. angustifolia* by Shukla et al. (2007), have showed 6.9 ± 0.69 micro shoots per explants within 6 week on MS medium supplemented with 3 mg/l BAP and 25 mg/l Ads whereas the present study exhibited a high frequency of shoot induction of 14.1 ± 0.55 shoots per explants with MS medium fortified with BA (13.3 µM), IAA (5.7 µM) and Ads (135.7 µM) within 60 days of inoculation. But the Ads concentration beyond 135.7 µM did not show any increase in the shoot bud proliferation. However, BA concentration beyond 13.3 µM did not manifest any increase in the micro shoot induction. In comparison to only cytokinins (BA or Kin) supplementing medium, shoot multiplications were enhanced on culture medium containing

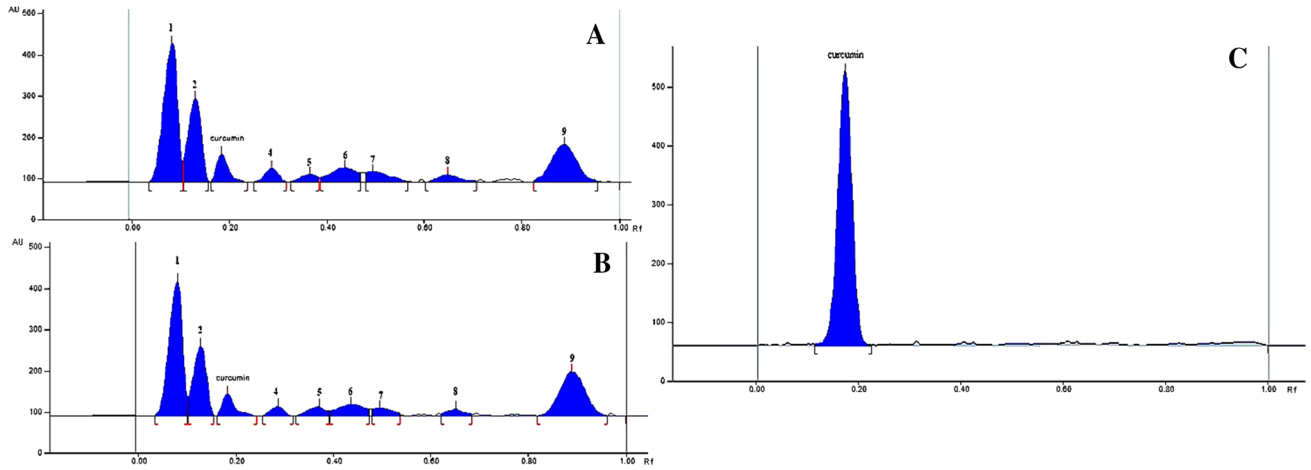

Fig. 4 HPTLC chromatogram at 254 nm (**A**) conventionally propagated extract of *C. angustifolia* (**B**) micropropagated extract of *C. angustifolia* (**C**) Standard curcumin

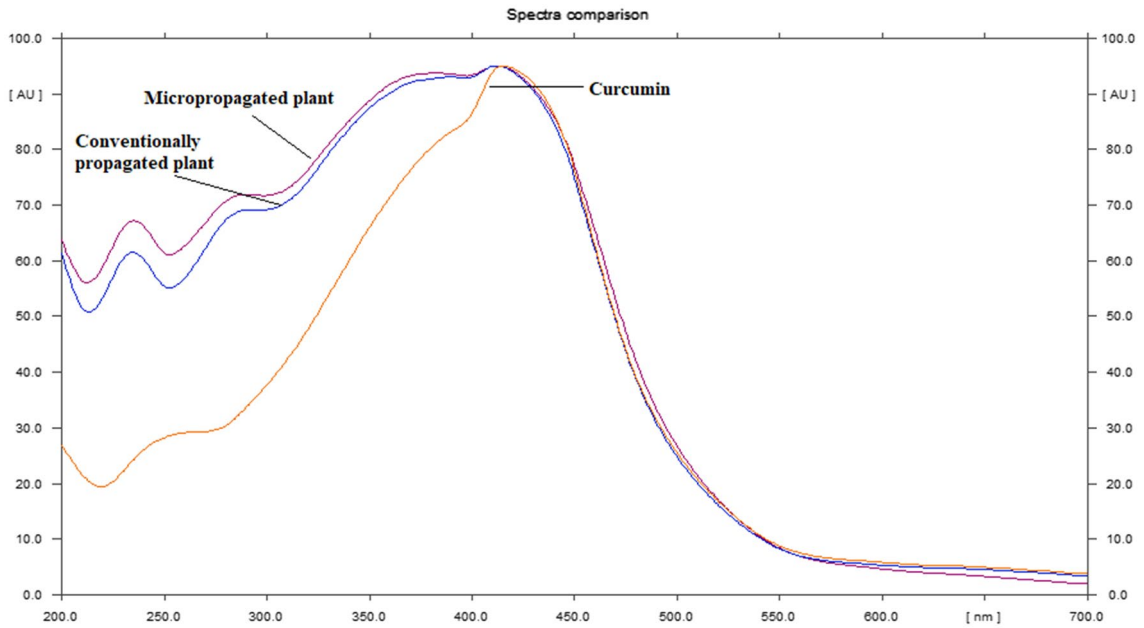

Fig. 5 Overlay of UV absorption spectra of conventionally propagated extract of *C. angustifolia* and micropropagated extract of *C. angustifolia* with standard curcumin

various concentrations of cytokinins (BA and Ads) and auxin (IAA). The culture medium when additionally supplemented with IAA (5.7 µM) displayed synergistic effect by inducing shoot number. Addition of Ads at optimum concentration of (135.7 µM) showed better growth and multiplication. According to Ahmad et al. (2018), Adenine in the form of Ads can promote cell growth as well as shoot bud formation. However, further increase in Ads concentration of medium had adverse effects. The role of Ads in enhancing proliferation was also reported in several medicinal plants such as *Carissa caranda* (Imran et al. 2012), *Syzygium cumini* (Naaz et al. 2014) and *Decalepis salicifolia* (Ahmad et al. 2018).

Essential oils of *C. angustifolia* are rich source of terpenic compounds having plethora of biological activities (Afzal et al. 2013; Sun et al. 2017). Our study is an agreement with Purkayastha et al. (2006) where curzerenone has been identified as the predominant compound in the rhizome oil of *C. zedoaria*. Curzerenone has been reported to show various biological activities, such as antimicrobial,

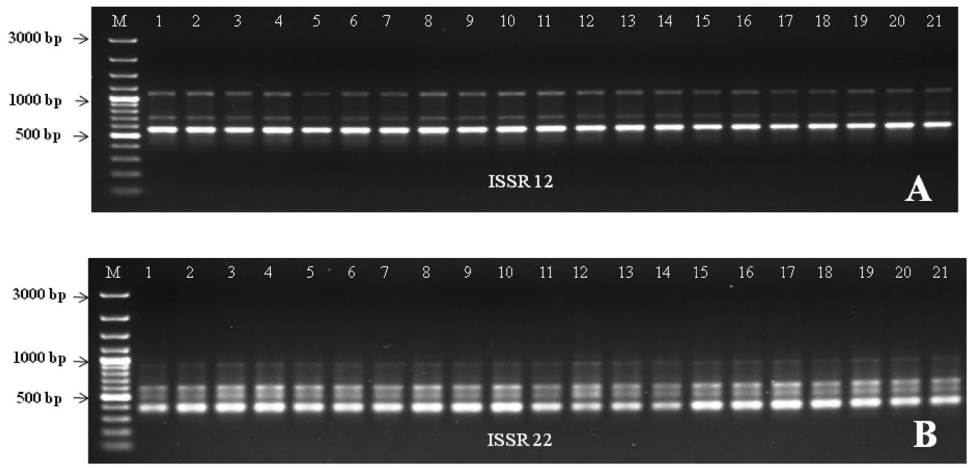

Fig. 6 Representative ISSR (**A, B**) profile of micropropagated and field grown mother plants of *C. angustifolia* (Lane 1: mother plant, Lane 2–21: randomly selected twenty micropropagated plants)

Table 4 ISSR banding patterns of micropropagated and conventionally grown plants of *C. angustifolia*

No.	Primer name	Primer sequence (5′–3′)	Total no. of bands amplified	No. of scorable bands per primer	Range of amplicons (bp)
1	ISSR 7	GACGACGACGACGAC	105	5	700–2000
2	ISSR 9	GTGTGTGTGTGTGTGTA	126	6	500–2300
3	ISSR 10	GTGTGTGTGTGTGTGTT	105	5	800–2100
4	ISSR 11	AGAGAGAGAGAGAGAGC	147	7	350–1700
5	ISSR 12	AGAGAGAGAGAGAGAGG	63	3	550–1100
6	ISSR 14	GAGAGAGAGAGAGAGAA	105	5	350–1200
7	ISSR 17	CTCTCTCTCTCTCTCTG	84	4	600–1700
8	ISSR 18	CACACACACACACACAT	84	4	800–1800
9	ISSR 19	CACACACACACACACAA	105	5	750–1800
10	ISSR 20	GTGGTGGTGGTGGTG	168	8	550–1800
11	ISSR 22	TGAGAGAGAGAGAGAGAGA	84	4	350–950
12	ISSR 23	GACAGACAGACAGACA	84	4	900–1600
Total			1260	60	350–2300

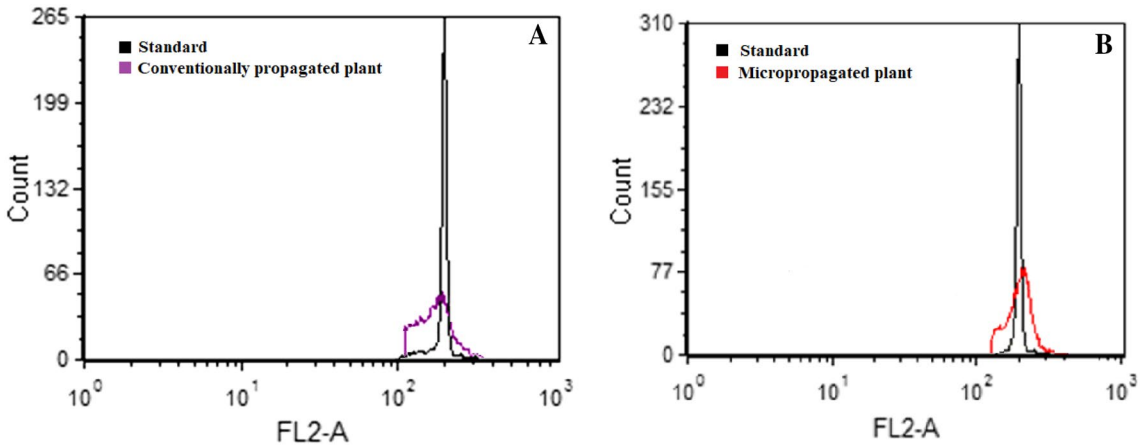

Fig. 7 Histogram of relative 2C DNA content of nuclei obtained from (**A**) conventionally propagated and (**B**) micropropagated plant of *C. angustifolia* with respect to internal standard (*Glycine max*)

anti-inflammatory, irritant, antioxidant and anticancer activities (Hsu 1980; Makabe et al. 2006; Xiang et al. 2011; Joshi and Mathela 2012). Our report is in contrary to the report of Srivastava et al. (2006) who reported xanthorrhizol isomer, methyl eugenol and palmitic acid as the major constituents in the rhizome oil from Central India plants, while the oil from Travancore plants was rich in germacrone, camphor and isoborneol. The remarkable differences in the constituents are likely due to geographical variation, edaphic or environmental factors (Djouahri et al. 2015; Jena et al. 2017; Ray et al. 2018). Further our results showed similarity in essential oil yield and constituents between micropropagated and conventionally propagated plants. Previous report by Mohanty et al. (2011a, b) in *Kaempferia galanga* rhizome oil has shown similarity in chemical constituents in somaclones and the source plant. Similarly, the higher uniformity of chemical components was found in leaf and rhizome essential oil in tissue culture derived plants of *Curcuma longa* to the conventional field grown plants (Nayak et al. 2011; Singh et al. 2011). No remarkable changes in the percentage of essential oil yield, curzerenone content and other identified constituents in leaf and rhizome oil between micropropagated and conventionally propagated plants of *C. angustifolia* were found, thus indicating the stability in phytomedicinal yield potential of regenerants.

Qualitative chemical uniformity between the in vitro regenerants and conventionally propagated plants of *C. angustifolia* was confirmed using HPTLC profiling. The HPTLC fingerprint pattern of chromatogram at $\lambda = 254$ nm was similar in in vitro and field grown plants. HPTLC analysis of rhizome extract revealing similar fingerprint profile and also confirmed chemical uniformity between tissue culture derived plantlets and conventionally propagated plant. This confirms that the in vitro plants were all true-to-type. The developed HPTLC fingerprint profile can be used as a reference fingerprint for the standardisation of *C. angustifolia* rhizome extract. HPTLC analysis also revealed the presence of curcumin. Curcumin is a biologically active component extracted from rhizomes of several *Curcuma* species, having potent biological activities (Lin and Lin-Shiau 2001; Woo et al. 2003; Anand et al. 2008; Cikrikci et al. 2008). Previously, the similarity in HPTLC fingerprinting profile in tissue culture and field grown plants have been reported by several researchers in several plants such as *Piper nigrum* (Ahmad et al. 2013), *Celastrus paniculatus* (Martin et al. 2006), *Asparagus adscendens* (Mehta and Subramanian 2005) and *Nothapodytes nimmoniana* (Prakash et al. 2016). Similarly, biochemical characterization of tissue cultured plants has also been documented by many researchers revealing stability in phytomedicinal yield potential (Nayak et al. 2011; Mohanty et al. 2011b; Singh et al. 2011).

ISSR marker based genetic integrity of in vitro regenerants have been documented in several plants such as *Kaempferia galanga* (Mohanty et al. 2011b), *Zingiber rubens* (Mohanty et al. 2011a), *Gerbera jamesonii* (Bhatia et al. 2009) and *Swertia chirayita* (Joshi and Dhawan 2007). ISSR analysis of in vitro regenerated plants of *C. angustifolia* showed similar profile to that of the mother plant showing that no genetic variation had occurred in vitro. Genetic stability of micropropagated *C. angustifolia* has also been confirmed by flow cytometry. Flow cytometry technique, has been used in plant sciences, mainly focused on nuclear DNA content, ploidy and genome size stability of micropropagated plants (Dolezel and Bartos 2005; Viehmannova et al. 2016). Analysis of the DNA content of conventionally propagated and plant regenerated from axillary bud by using flow cytometry analysis confirmed unalteration in nuclear DNA content of *C. angustifolia*. From the earlier report, it has been documented that the 2C DNA content of the *C. angustifolia* collected from Bangladesh were found to be 2.121–2.141 pg (Islam 2004). Similarly, genome size stability has been documented in several in vitro regenerants as well as medicinal plants like *Hydrastis Canadensis* (Obae and West 2010), *Solanum trilobatum* (Shilpha et al. 2014), *Puya berteroniana* (Viehmannova et al. 2016) and *Solanum lycopersicum* (Alatar et al. 2017). The present findings confirmed that the established protocol guarantees in vitro regeneration of plants with stable genome size.

In conclusion, the present protocol of rapid plant regeneration using axillary bud explants with genetically uniform plantlets and stable phytomedicinal yield potential can be recommended for effective mass propagation of true-to-type *C. angustifolia* (East Indian Arrowroot) for commercial utilization.

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