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Original Article

EVALUATION OF DRUG YIELDING POTENTIAL OF MICROPROPAGATED *CURCUMA AROMATICA*

SUJATA MOHANTY, REENA PARIDA, I SRIRAM SANDEEP, SUPRAVA SAHOO, SANGHAMITRA NAYAK*

Centre of Biotechnology, Siksha O Anusandhan University, Kalinga Nagar, Ghatikia, Bhubaneswar 751003, Odisha, India.
Email: sanghamitran24@gmail.com

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ABSTRACT

Objective: GC MS analysis and antioxidant activity of micropropagated and conventionally grown *Curcuma aromatica* essential oil and extract was done for their large scale commercial cultivation. Molecular marker based studies were performed to know their genetic fidelity as well as to trace any somaclonal variation existing between the regenerants.

Methods: *In vitro* regeneration and multiplication were done using Murashige and Skoog media with various combinations of growth regulators. Component identification was done by GC MS analysis. Random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) markers were used for molecular profiling. Antioxidant activity was performed using 2, 2-diphenyl-1-picrylhydrazyl (DPPH).

Results: Molecular marker-based analysis revealed uniform banding patterns similar to those of the mother plants. Gas chromatography and mass spectroscopy (GC MS) analysis showed the presence of 10 major components accounted for 95.5% of the total compounds. The major components in micropropagated and field grown mother plants were found to be alpha phellandrene (41% and 38%), 4-carene (23% and 25%) and terpeneol etc. Antioxidant activity of leaf oil (IC_{50}-29.3 µg/ml) and methanolic extract (IC_{50}-101.25 µg/ml) of *in vitro* grown plants showed increased free-radical scavenging activity.

Conclusion: Absence of any type of remarkable polymorphism in the essential oil quality and antioxidant activity the present protocol could be used commercially for large scale propagation of *C. aromatica*. The present report bears immense potential for the future improvement, conservation and domestication of *C. aromatica* to explore its high prized secondary metabolites.

Keywords: *Curcuma aromatica*, Micropropagation, GC MS, RAPD, ISSR, DPPH

INTRODUCTION

Curcuma aromatica, commonly known as wild turmeric is an under exploited medicinal plant. The processed and dried rhizome is an important flavouring and coloring agent used in culinary and food preparations in many parts of the world. The rhizome is a rich source of volatile oil which is responsible for its pleasant aroma and the color is due to presence of its high content of curcumin. The rhizome is considered to be a tonic and carminative and is also used to cure leucoderma. It is used externally in the treatment of scabies and smallpox; made in to a paste with benzene is applied on the forehead to obtain relief from headache [1]. The essential oil has been reported to have anti microbial and anti fungal activity and is used in the treatment of early stages of cervical cancer [2]. Recently, curcumin the active principle *C. aromatica* has been found to have anti carcinogenic properties and is now used in the formulation of an anticancer drug due to its inhibitory effect on the induction and growth of tumors [3]. The average productivity and quality are not satisfactory due to slow multiplication rate, overexploitation and habitat destruction which are probably the main drawbacks to meet the ever increasing market demand. Further, cultivation of these wild medicinally important plants lack sufficient disease free elite planting materials due to high susceptibility of the crop for rhizome rot, leaf spot and bacterial wilt [4]. These problems can be alleviated through the application of tissue culture technique, an efficient long-recognized tool for rapid multiplication of true-to-type genotypes [5-7].

In vitro culture of medicinal plant is always associated with the possible occurrence of somaclonal variation among the sub clones of potential lines which arise as a direct consequence of plant cell, tissue and organ culture [8]. Besides, the genetic control of phytoconstituents of these plants is another major problem associated with commercial micropropagation of medicinal plants to supply the market with crude drugs of uniform and stable quality. This necessitates the need for assessment of drug yielding potential of tissue culture derived wild turmeric in field by biochemical and DNA based molecular markers. Molecular analysis using DNA based markers has been reported in many plant species for *in vitro* monitoring of genetic stability [7, 9, 10] but no report is available on biochemical and molecular analysis of micropropagated plants of *C. aromatica* grown ex vitro. Few reports are available on metabolite analysis in wild turmeric but are confined mostly to analysis of morphological characteristics lacking detail study of their important drug yielding traits in relation to genetic stability assessment [11-13]. Besides, the leaf essential oil and extract of *C. aromatica* also posses antioxidant activity [13]. Antioxidants posses the ability to reduce the oxidative damage associated with many disease including neurodegenerative diseases, cancer, cardiovascular disease, cataracts and AIDS. Hence restoration of antioxidant activity in micropropagated clones should always associated with the evaluation of its antioxidant activity for large scale propagation and commercialization.

The aim of our present study is to make the comparative field evaluation of the drug yielding potential of tissue culture derived and conventionally grown *C. aromatica* co-relating with their potent antioxidant activity and genetic stability assessment through molecular marker analysis.

MATERIALS AND METHODS

Plant material collection and culture conditions *in vitro*

Rhizomes of *C. aromatica* collected from the High Altitude Research Station (HARS) Pottangi, Orissa, were grown in the medicinal plant garden of the Center of Biotechnology, Siksha O Anusandhan University, Bhubaneswar, Orissa (India). Sprouted buds of the rhizomes were used as explant and were thoroughly washed with water. These explants were then dipped in liquid detergent (Extran; Merck, Germany) for 3–5 min. These were then thoroughly washed to remove the last drop of detergent. Surface sterilization was done by 0.1% of mercuric chloride solution for 8–10 min. After sterilization, the explants were washed with sterile distilled water under aseptic conditions prior to inoculation. Explants were inoculated into the basal medium [14] containing varying

combinations of benzyl adenine (BA) (1–5 mg/l), indole-3-acetic acid (IAA) (0.5–1.0 mg/l), naphthalene acetic acid (NAA) (0.5 mg/l), gibberlic acid (GA) (0.5–1 mg/l) and kinetin (Kn) (0.5–1 mg/l) as listed in table 1. Fifteen replicates were used for each treatment. Prior to inoculation, MS media containing hormones were autoclaved at 121°C and 1.05 kg/cm2 of pressure for 20 min. The pH of the media was adjusted to 5.7. Culture tubes containing the inoculated explants were kept under white fluorescent light with 55 µmole m^{-2} s^{-2} light intensity. The percentage of response of those explants to different hormonal combinations was recorded. Culture conditions were similar to those used at the time of initial inoculation. *In vitro* regenerated plantlets having well-developed shoots and roots were transferred to pots containing soil and sand mixture in 1:1 ratio after 90 days of growth in culture. These were then kept in a greenhouse for acclimatization. After 4weeks, these were transferred to the normal field conditions and grown to maturity. After 6 months of transplantation, the morphological characters such as plant length, leaf biomass, leaf number, rhizome weight and percentage of leaf essential oil were recorded and compared with their wild grown mother plants. Coefficient of variation (CV) were calculated for each characters (CV = Standard Deviation/mean x 100).

DPPH radical scavenging assay

The free radical scavenging activity of *C. aromatica* leaf extract and leaf oil and ascorbic acid was measured in terms of hydrogen donating or free radical scavenging ability by using the stable 1, 1-diphenyl-2-picryl hydrazyl radical (DPPH) following the method[11]. 0.1 mM DPPH solution was mixed with various concentrations (10-500 µg/ml) of leaf extract and leaf oil of *Curcuma aromatica*. The mixture was shaken vigorously and incubated at room temperature for 30 min in the dark. The reduction of the DPPH free radical was measured by reading the absorbance at 517 nm by a spectrophotometer. The solution without any extract and with DPPH and methanol was used as control. The experiment was replicated three independent assays. Ascorbic acid was used as positive controls. Inhibition of DPPH free radical in percentage was calculated by the formula:

DPPH radical scavenging activity (%) = $[(A_{control}-A_{test})/A_{control}] \times 100$

Where, $A_{control}$ is the absorbance of the control (solution without extract) and A_{test} is the absorbance of samples (extract and ascorbic acid).

DNA extraction and ISSR, RAPD analysis

DNA was isolated from young leaves of *C. aromatica* following the protocol [16]. The quality and quantity of the DNA were determined with a Thermo Scientific UV-Vis spectrophotometer. RAPD and ISSR analysis was done following the standard protocol [17, 18]. Primers were selected on the basis of the clarity of banding patterns by repeating the process twice. The amplification was carried out in a thermal cycler (Gene Amp PCR system 9700; Applied Biosystems, CA, USA). Size of the amplicons was determined using size standards Gene ruler 100 bp DNA ladder plus (MBI Fermentas, Lithuania). DNA finger prints were photographed and documented using gel documenting system (Bio-Rad, USA).

Extraction of essential oil and GC–MS analysis

Essential oil of both tissue culture-derived and conventionally propagated plants was extracted by hydro-distillation in a Clevenger's apparatus following the method [19]. Prior to GC-MS analysis the essential oil was diluted with dimethyl sulphoxide (DMSO). The component identification was achieved by the GC–MS analysis using HP 6890 series GC (Hewlett-Packard, USA) coupled with a mass selective detector (MSD), HP 5973 series (Hewlett-Packard). Helium was used as a carrier gas and the sample was injected in split less mode in a column HP5 phenyle methyl siloxane [25 µm (film thickness) x 320 lm (internal diameter) x 30 m (length of column)]. Mass spectra were acquired over a 40–400 atomic mass unit range. Compounds were identified by comparing the mass spectral data with those in the NIST library provided with software and with commercially available data. Temperature programming was: initial temperature 60°C, ramping rate 3°/C, and final temperature 243°C, run time 61 min. For GC–MS evaluation, 15 plants from micropropagated and wild grown were randomly selected.

The rhizomes from both field grown and micropropagated plants were dried in shade condition and were powdered to uniform mesh. Estimation of curcumin was done following ASTA procedure [20, 21]. The absorption maxima of curcumin were measured at 425 nm using a spectrophotometer (Thermo Scientific, India). Percentage of curcumin was calculated taking pure curcumin (98%) as standard [22]. Analysis of curcumin was done in triplicates up to three generations for micropropagated plants and average was taken for data analysis.

RESULTS

***In vitro* multiplication and morphological characteristics**

Freshly sprouted shoots of *Curcuma aromatica* excised at the base from the rhizomes of field grown plants were cultured on MS basal medium supplemented with BA alone or with different combinations of BA, IAA, NAA and kinetin. MS media with BA (5 mg/l) showed 87% of in vitro shoot initiation. Two to five lateral shoots developed from the basal region within 20-25 days of culture. The medium supplemented with 3 mg/l BA was the most effective for multiple shoot formation producing an average of 11.2±0.3 numbers of shoots and 6.4±0.2 no of roots per explants (table 1). The multiplication rate did not change during the subsequent subculture up to 2 years. MS media with BA 5 mg/l was showed less number of shoots. Other media combination with NAA and kinetin showed less response. Kinetin alone was not enough to produce shoot in *C. aromatica*. Cultured plantlets were successfully established in field after 3 weeks of acclimatization in the green house. Morphological characteristics and essential oil content of both tissue culture and wild grown mother plants were compared and recorded up to three successive generations (table 2). Morphology and essential oil percentage in both the plants were mostly similar with very less difference in them. Analysis of curcumin was by taking rhizomes from both field grown and micropropagted plants. Average curcumin percentage in field grown conventional plants was found to be 3.6 whereas in micropropagated clones it was 4.5%. The percentage of curcumin was remain constant up to three successive generations (table 2).

Table 1: *In vitro* shoot multiplication of *C. aromatica* on MS medium fortified with different growth regulators

S. No.	MS media with growth regulators (mg/l)				% of shoot initiation	No of shoots per explants (mean±SE)	No of roots per explants (mean±SE)
	BA	IAA	NAA	Kn			
1	1	-	-	-	61.8±0.4	9.3±0.2	4.2±0.4
2	3	-	-	-	71.8±0.5	11.2±0.3	6.4±0.2
3	5	-	-	-	87.2±0.8	3.6±0.2	2.4±0.4
4	7	-	-	-	61.8±0.4	2.4±0.3	1.8±0.5
5	1	0.5	-	-	41.6±0.6	2.2±0.2	1.8±0.2
6	3	0.5	-	-	66.6±0.5	4.2±0.2	1.8±0.5
7	1	-	0.5	-	41.8±0.8	2.4±0.3	1.0±0.3
8	3	-	0.5	-	56.8±0.5	1.0±0.3	2.8±0.2
9	5	-	0.5	-	37.0±0.5	0.8±0.4	1.4±0.4
10	1	0.5	-	0.5	62.2±0.6	2.4±0.3	0.8±0.3
11	3	1	-	0.5	73.2±0.5	3.0±0.3	2.6±0.2
12	1	-	0.5	0.5	54.0±0.6	2.6±0.3	1.4±0.4
13	3	-	1	0.5	66.8±0.5	4.2±0.5	3.2±0.5

Table 2: Comparison of different morphological and biochemical characteristics between *in vitro* grown plants and wild grown mother plants of *C. aromatica*

Character	Wild grown mother plants		*In vitro* grown plants	
	mean±SD	CV (%)	mean±SD	CV (%)
Plant length (cm)	142±2.08	1.46	157±0.78	0.49
Leaf biomass(gm)/ plant	135±2.63	1.94	152±0.56	0.36
No. of leaves/plant	10±0.85	8.5	12±0.34	2.8
Rhizome yield (gm)/ plant	83±2.03	2.44	106±0.87	0.82
Leaf oil (%)	0.4±0.03	7.5	0.6±0.02	3.33
Rhizome oil (%)	0.6±0.05	8.3	0.7±0.02	2.8
Curcumin (%)	3.5±0.03	0.85	4.0±0.03	0.75

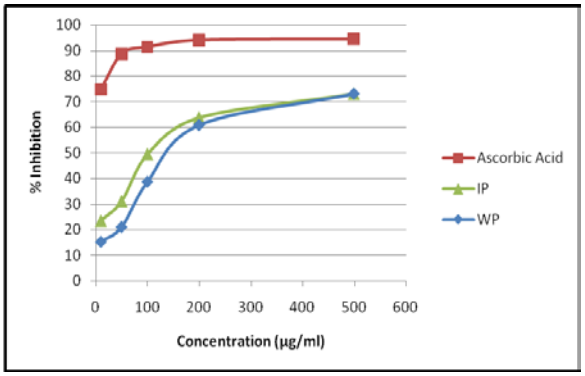

Fig. 1: DPPH radical scavenging activity of leaf essential oil of *in vitro* and wild grown *Curcuma aromatica.* (IP-*in vitro* grown plant, WP-wild grown plant)

DPPH activity

The free radical scavenging activity was expressed as the effective concentration required for 50% of DPPH radical reduction (IC_{50}) obtained from a plot of the graph of scavenging activity against the concentration of the extract and oil. Fig. (1 and 2) shows the IC_{50} (µg/ml) values of plant extracts and essential oils for free radical scavenging activity by DPPH radical. The IC_{50} value of *C. aromatica* leaf extract was 101.25 µg/ml while the IC_{50}value of standard antioxidant ascorbic acid was 6.58 µg/ml. Among the oil and extract, the methanolic extract has profound reducing activity against stable free radicals.

GC – MS analysis

Essential oil from leaves of micropropagated *C. aromatica* and its wild grown mother plants was subjected to GC – MS analysis to evaluate its detailed chemical composition. The analysis showed 10 major identified compounds accounting for 98.3% and 92.3 % of the total peak area for tissue cultured and its wild grown mother plants respectively (table 3). Compounds were identified by comparing the mass spectral data with those in the NIST Wiley library provided with software and with commercially available data. The compounds present in both cases were quite similar to each other. α-phellandrene was the major compound in both wild grown and micropropagated clones of *C. aromatica*, comprised the maximum peak area (41.3 %) followed by 4-carene, γ-terpenene, tumerone, terpeneol, toluidine, and farnesene etc.

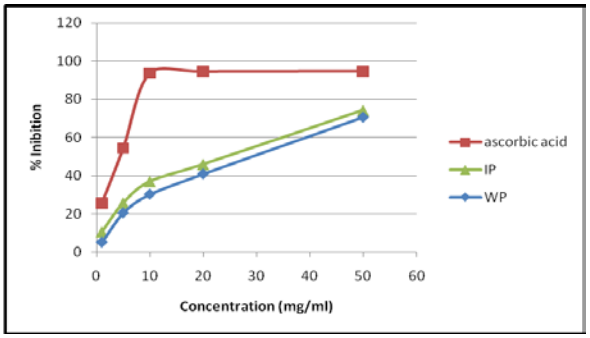

Fig. 2: DPPH radical scavenging activity of leaf extract of *in vitro* and wild grown *Curcuma aromatica.* (IP-*in vitro* grown plant, WP-wild grown plant)

Table 3: Quality evaluation of essential oil from leaves of wild grown mother plants and *in vitro* grown *C. aromatica*

Oil constituents	KI	Wild grown mother plants mean±SD	*In vitro* grown plants mean±SD
α-phellandrene	1005	38.54±0.35**	41.3±0.24**
γ-terpenene	1055	17.95±0.40**	20.16±0.31**
4-carene	1001	25.49±0.50***	23.08±0.35***
4-terpinenol	1198	1.43±0.72***	1.17±0.15***
cadinene	1538	0.42±0.67**	0.5±0.65**
β-farnesene	1650	0. 77±0.27***	1.47±0.05***
Tumerone	1664	5.66±0.25**	7.34±0.03**
p-toluidine	1380	1.56±0.83***	1.87±0.63***
Curlone	1650	0.45±0.07***	1.04±0.72***
Benzyl alcohol	1313	----------	0.81±0.39***

KI Kovat Index, Data were significant at ** P = 0.02 and *** P = 0.05

ISSR and RAPD analysis

A total of 60 plants were analyzed in *C. aromatica* upto 3 years with an interval of six months taking minimum 10 plants at a time. Out of 25 RAPD primers used, 19 primers gave a total of 67 scorable bands ranging from 200-1900 bp. Number of bands varied from 1-10 with an average of 3.5 per primer. A total of 4020 bands [(number of plantlets analyzed) x (number of bands with all primers)] generated by the RAPD techniques showed monomorphic patterns across all 60 plantlets analyzed. Highest number of monomorphic band was 10 in primer OPC11 (ranging from 600-1900bp) where as lowest number of band was 1 in primer OPA7 (1475bp), OPD12 (900bp) and OPAF15 (650bp). In ISSR analysis 8 primer gave rise to a total of 58 bands ranging from 250-1500bp which were all monomorphic without any variations. Number of bands in all primers varied from 5-11 with an average of 7.2 bands per primer. A total of 3480 bands

[(number of plantlets analyzed) x (number of bands with all primers)] were generated by the ISSR techniques, giving rise to monomorphic patterns across all 50 plantlets analyzed, highest 11 in primer (GGA) 4(ranging from 300-1500bp) and lowest in (GTGC) 4(ranging from 300-1050bp) and T(GA)9 (ranging from 600-1100bp) (table 4).

Table 4: RAPD and ISSR banding pattern of micropropagated and field-grown mother plants of *C. aromatica*

	Primers	Sequences	Total number of bands	Range of size of the amplicons [bp]
RAPD	OPA4	AATCGGGCTG	4	600-1500
	OPA7	GAAACGGGTG	1	1475
	OPA9	GGGTAACGCC	2	1200-1400
	OPA18	AGGTGACCGT	5	200-1075
	OPC2	GTGAGGCGTC	5	650-1050
	OPC5	GATGACCGCC	2	1700-1900
	OPC11	AAAGCTGCGG	10	600-1900
	OPD3	GTCGCCGTCA	2	1031-1350
	OPD7	TTGGCACGGG	5	450-1250
	OPD8	GTGTGCCCCA	3	1031-1800
	OPD12	CACCGTATCC	1	900
	OPD18	GAGAGCCAAC	2	750-1031
	OPD20	ACCCGGTCAC	7	400-1900
	OPN4	GACCGACCCA	3	500-1900
	OPN16	AAGCGACCTG	5	300-1200
	OPN18	GGTGAGGTCA	4	450-1050
	OPAF5	CCCGATCAGA	2	200-1050
	OPAF14	GGTGCGCACT	3	900-1200
	OPAF15	CACGAACCTC	1	650
	Total 67			
	SPS 1	(GAC)5	9	350-1031
	SPS 2	(GTGC)4	5	300-1050
	SPS 3	(GACA)4	8	475-900
	SPS 4	(AGG)6	6	400-1031
	SPS 5	(GA)9T	6	450-975
	SPS 6	T(GA)9	5	600-1100
	SPS 7	(GTG)5	8	250-850
	SPS 8	(GGA)4	11	300-1500
ISSR	**Total 58**			

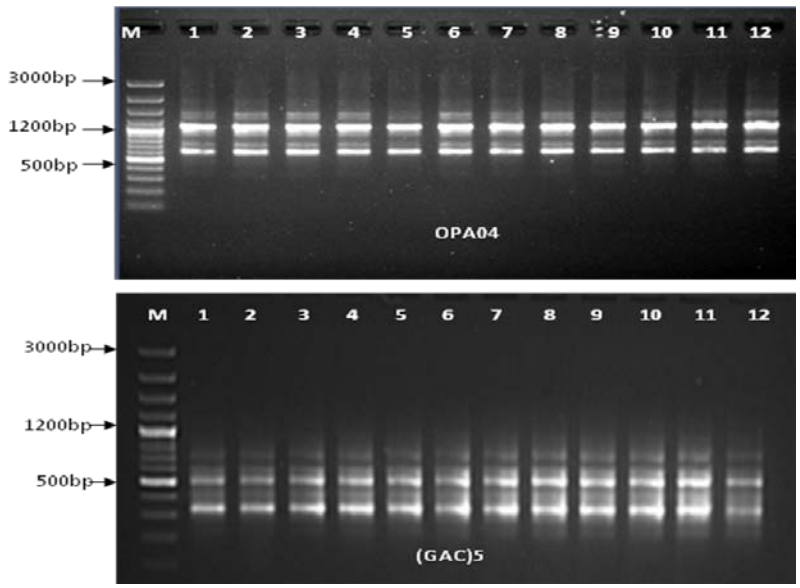

Fig. 3: RAPD (OPA4) and ISSR [(GAC) 5] marker profiles of *in vitro* (1-2) and wild grown (3-12) *Curcuma aromatic*

DISCUSSION

Micropropagation of *C. aromatica* has been reported [23-25]. In other reports 14 numbers of shoots were found where as in our report it was 11 [23]. This may be due to the genotype and geographical situation of the source plants. Further in our case though the number of shoots was 11 but initiation of roots occurred simultaneously in the same media. Hence the regenerated shoots needn't have to transfer to rooting media which minimize the time of propagation and media cost. In any *in vitro* regenerated plants besides the pre existing variation, somaclonal variation leads to phenotypic variation in the explants which is either directed by the organism or induced by stress [26, 27]. To detect the occurrence of any phenotypic variation in the in vitro propagated *C. aromatica,* morphological characteristics like plant height, tiller number, leaf biomass etc were measured and compared to their wild grown

mother plant. The regenerants showed similar morphological profile as their mother plants but were more uniform with a higher CV value. Our report is in close agreement with reports of other species such as *Curcuma longa* and *Kaempferia galanga* where the micropropagated clones were more uniform by means of their volatile constituents [28, 29]. High uniformity in essential oil content of culture derived clonal plants of *Cymbopogon flexnosus* compared to the field cultivated clones was reported [30]. Similar report is available on high uniformity in alkaloid content of culture derived somaclones of *Aconitum carmicheli* as compared to the field cultivated clones [31]. As *C. aromatica* is a vegetatively propagated plant the stable morphological and biochemical parameters were carried out up to the third generation studied.

According to others, *in vitro* studies should always accompanied with anti oxidant activity due to the variation in secondary metabolites and their constituent leading to complex structure-activity relationship [32]. Thus the present study on anti oxidant activity of leaf essential oil and extract showed similar profile in both micropropagated as well as wild grown plants (fig. 1 and 2). Antioxidant activity of micropropagated *Poliomintha glabrescens* plants was reported to have similar activity as that of their wild grown mother plants [33]. The oil and methanol extract showed potent DPPH radical-scavenging activities (IC (50) =14.45 and 16.58 µg/ml, respectively. The radical scavenging activity in *Aloe arborescens* also increases with the increase concentration of cytokinin as reported by others but in our case we have chosen only the best media according to the response which also optimized for the production of stable bioactive secondary metabolites [34]. The antioxidant activity of in vitro propagated plants was shown to have similar activities when compared with the field grown plants. Hence the radical scavenging activity which is one of the important medicinal properties of Zingiberaceous plants could be restored through tissue culture for large scale commercial propagation.

In micropropagated clones, there is always a chance of variation in their morphological or biochemical characteristics. To detect variation among the regenerants and their field grown mother plants, these were subjected to GC-MS analysis. GC-MS based quality and quantity evaluation of essential oil from micropropagated clones were compared with the field grown plants. Essential oil of leaves contain alpha phellandrene as the major compound in both micropropagated (41.3%) and field grown plants (38.5%). Our report is contrary to others who reported camphor (28%) and 1, 8-ceneole (20%) were the major constituent of *C. aromatica* leaf essential oil [11, 12]. This variation may occur due to climatic, geographic or genotypic differences which could be responsible for the differences in the chemical composition of essential oil.

The possible occurrence of genetic variation in micropropagated plants are due to cytogenetic abnormalities and specific sequence changes which can be analyzed by RFLP, RAPD, ISSR and SSR analysis [35-38]. Thus micropropagated clones of *C. aromatica* with stable drug yielding potential were subjected for RAPD and ISSR analysis for their genetic fidelity assessment upto 3 years with a regular interval of six months in culture. The amplified bands showed uniform banding pattern in both control and in vitro regenerated plants. RAPD and ISSR markers were successfully used to evaluate the genetic fidelity as well as variation of other micropropagated plant species [39-42]. Absence of any type of remarkable polymorphism in the essential oil quality and antioxidant activity the present protocol could be used commercially for large scale propagation of *C. aromatica* [43, 44]. The stable drug yielding potential of the micropropagated plants were further supported by their uniform genetic fingerprints as compared to the field grown mother plants. Thus the present report bears immense potential for the future improvement, conservation and domestication of *C. aromatica* to explore its high prized secondary metabolites.

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CONFLICT OF INTERESTS

Declared None

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