

In vitro and ex vitro evaluation of long-term micropropagated turmeric as analyzed through cytophotometry, phytoconstituents, biochemical and molecular markers

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Abstract Turmeric (*Curcuma longa* L.), a high valued medicinal plant, was micropropagated through induction of multiple shoots using latent axillary buds of rhizome. Cytophotometric and random amplified polymorphic DNA (RAPD) as well as inter simple sequence repeats (ISSR) analysis were used to periodically monitor the genetic stability of micropropagated clones of *Curcuma longa* conserved in vitro up to 7 years at every 6 months interval. A total of eighteen RAPD and eight ISSR primers gave 45,537 distinct and reproducible bands, monomorphic across all 353 plants analyzed. Micropropagated turmeric after being conserved for 7 years in vitro was transplanted into soil in field. Drug yielding potential of tissue culture derived plants was evaluated in field through estimation of phytoconstituents like curcumin and essential oil contents. The result of 2 years of field trial showed that micropropagated turmeric retained stability in all the characteristics examined when compared with the field performance of conventionally propagated plants. Thus long term conservation of an elite genotype of turmeric with epigenetic and genetic stability is significant for stable supply of drug i.e., curcumin and essential oil to the market.

Keywords *Curcuma longa* · Drug yield · Micropropagation · Stability

Introduction

Tissue culture technique has long been recognized as an efficient tool for rapid clonal multiplication and conservation of desirable genotypes (Hussey 1986; Ashmore 1997; Tyagi et al. 2004, 2006). For commercial utilization of in vitro propagation technique, it is necessary to raise enough disease free planting material, true-to-type of newly developed elite lines that are available in small quantities. A major problem associated with in vitro culture is possible occurrence of somaclonal variation arising in culture regenerated plantlets and their progenies (LarKns and Scowcroft 1981; Gould 1986). Assessment of degree of genetic integrity of micropropagated plants at regular interval can reduce the chances of induction of somaclonal variation that might arise due to length of culture period (Rout and Das 2002; Mohanty et al. 2008, 2010; Nayak and Sen 1995). Of several methods phenotypic analysis, chromosome analysis, estimation of nuclear DNA content, analysis of secondary biochemical products are used for detection of somaclonal variation among the regenerants (Potter and Jones 1991; Nayak and Sen 1991, 1997). Molecular analysis is being widely used for monitoring genetic stability of in vitro raised and conserved materials. DNA based markers provide an efficient way to screen the tissue culture induced variations as these markers are not affected by environmental factors (Peredo et al. 2009). Some of the molecular techniques used to detect genetic variation are polymerase chain reaction (PCR)- based markers such as random amplified DNA polymorphism (RAPD), inter simple

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sequence repeats (ISSR) and amplified fragent length polymorphism(AFLP), methylation sensitive amplified polymorphism (MSAP) and retrotransposon microsatellite amplified DNA polymorphism (REMAP). These multilo- cational profiling techniques are powerful tools for assessment of genetic stability of regenerants after micropropagation (Martins et al. 2004; Mohanty et al. 2008, 2010; Morata-Renau et al. 2005; Peredo et al. 2009; Smykal et al. 2007).

No single technique being ideal or sufficient, taken alone, for the assessment of somaclonal variation, a combination of several techniques should be used to evaluate the micropropagated plants (Peredo et al. 2009). In the present paper, cytophotometric, phytochemical and molecular analysis were used to evaluate micropropagated *Curcuma longa*.

Curcuma longa L., commonly known as turmeric is valued worldwide for its medicinal properties (Roses 1999). Turmeric posses several pharmacological activities like anti-inflammatory, hepatoprotective, anti-microbial, wound healing, anti- cancer, anti-tumor, anti-viral and anti-malarial properties. The drug yielding potential of turmeric is due to the presence of curcumin, oleoresin and essential oil in rhizome and leaf. Slow rate of multipli- cation, limited availability of elite genotypes, expensive field maintenance of planting material and high suscep- tibility of turmeric to rhizome rot diseases necessitate application of tissue culture techniques for stable supply of disease free elite planting materials of turmeric. The genetic control of biochemical and morphological char- acteristics of plants remains as one of the major problems associated with commercial production of medicinal plants by tissue culture to supply the market with crude drugs of uniform and stable quality. This problem necessitates the evaluation of drug yielding potential of micropropagated plants (Hatano et al. 1988). Furthermore, in order to ascertain the stability of drug yielding traits, field evaluation needs to be carried out in subsequent generations.

Surama is an elite cultivar of turmeric with high rhizome yield and improved drug yielding potential. All available papers and reviews dealing with studies on tissue culture of *Curcuma longa* exclude detailed analysis of genetic stability of long term in vitro conserved plantlets and subsequent *ex vitro* evaluation of their drug yielding traits. (Nadgauda et al. 1978; Balachandran et al. 1990; Panda et al. 2007; Salvi et al. 2002; Nayak and Naik 2006; Xiaoqiang and Gang 2006). In the present paper, we have reported retention of genetic stability among in vitro con- served turmeric (cv. Surama) up to 7 years and subsequent field evaluation for 2 years revealing stable drug yielding potential.

Materials and methods

Plant material

Rhizome of *Curcuma longa* (cv. Surama) was collected from the High Altitude Research Station, Pottangi and was grown in medicinal plant garden of Centre of Biotechnol- ogy, Bhubaneswar, Orissa (India). Dormant axillary buds of unsprouted rhizomes were used as explants and were thor- oughly washed with water. These explants were kept under running water for 10–15 min and then dipped in liquid detergent (Extran, Merck, Germany) for 3–5 min. These were thoroughly washed with distilled water. Surface decontamination was done by 0.1% mercuric chloride solution for 8–10 min. After decontamination the explants containing buds were washed several times with sterile distilled water under aseptic condition prior to inoculation.

Establishment of in vitro culture

Explants were inoculated to the basal medium of Murashige and Skoog (MS) (1962) with combination of 1–5 mg/l of 6-Benzylaminopurine (BA), 1–2 mg/l of Indole-3-Acetic Acid (IAA), 1–2 mg/l of 1-Napthalene Acetic acid (NAA), 1–2 mg/l of Kinetin (Kn) and 50–100 mg/l of Adenine sulphate (Ads). Fifteen replicas were used for each treatment for recording data on percent of explants forming shoots, number of shoots per explants and formation of roots. Prior to inoculation, MS media containing plant growth regulators were autoclaved at 121°C and 1.2 kg/cm² of pressure for 20 min and pH of the media was adjusted to 5.7. Culture tubes containing the inoculated explants were kept under white florescent light with 50 $\mu\text{M m}^{-2} \text{ s}^{-1}$ light intensity. A single explant was inoculated on 20 ml medium in 50 ml glass culture tube plugged with cotton plug. Micropropagated plantlets were maintained with regular subculturing at 90 days interval. For better rhizome yield, the plantlets were transferred to the micro-rhizome induction media and cultured with a reduced photoperiod of 4 h daily following our earlier established protocol (Nayak and Naik 2006).

Cytophotometric analysis

For analysis of the 4C nuclear DNA content through cyto- photometry, root tips of in vitro grown regenerants were collected aseptically from culture tubes and were fixed over night in propionic acid/ethanol (1:3). This treatment was followed by hydrolysis in 1 N HCl (v/v) at 60°C for 12 min. The root tips were washed in distilled water, stained in Schiff's reagent for 2 h at 14°C and squashed with 45% acetic acid. The DNA content of nuclei was measured with

a Leitz Wetzlar microspectrophotometer using the single wavelength (550 nm) method (Sharma and Sharma 1980). In situ DNA values were obtained on the basis of optical densities which were converted to picograms (pg) by using Van't Hof's 4C nuclear DNA value of *Allium cepa* (67.1 pg) as standard (Van't Hof 1965).

RAPD and ISSR analysis

RAPD and ISSR analysis was carried out using in vitro grown leaves of *C. longa* up to seven years at an interval of six months. The RAPD analysis was performed as per the method of Williams et al. (1990) and for ISSR analysis the method of Zeatkiewicz et al. (1994) was followed. To isolate and purify the genomic DNA from field grown plant for RAPD analysis the protocol of Doyle and Doyle (1990) was followed with a little modification i.e. samples were pulverized with 2% polyvinyl polypyrrolidone (PVPP). Quantification of DNA was accomplished by analyzing the purified DNA on 0.8% Agarose gel alongside uncut lambda DNA as standard. DNA was diluted in T₁₀E₁ to 25 ng/μl for PCR analysis. A total of 30 random primers were utilized for RAPD analysis, out of which 18 random decamer primers (Operon Tech., Alameda, USA) from A, C, D and N series were used. For ISSR analysis 8 out of 10 primers were selected on the basis of the clarity of banding patterns. DNA amplification was performed in thermal cycler (Applied Biosystems, model gene amplification PCR system 9700, USA) in a volume of 25 μl containing 25 ng of template DNA, 2.5 mL of 10× assay buffer (100 mM Tris–Cl, pH 8.3, 500 mM KCl, and 0.1% gelatin), 15 mM MgCl₂, 200 μM of each dNTPs, 15 ng of primer and 0.5 unit of Taq DNA polymerase (Bangalore Genei Pvt. Ltd, Bangalore, India). Following an initial denaturation step at 92°C, 1 min at 37°C and 2 min at 72°C for 42 cycles, amplification products were separated by electrophoresis on 1.5% agarose gel in 1× TAE buffer stained with ethidium bromide and visualized with uv light. The size of the amplicon was determined using size standards Gene Ruler 100 bp DNA ladder plus (MBI Fermentas, Lithuania). DNA finger prints were photographed using gel documenting system. RAPD and ISSR analyses using all primers were repeated at least twice to establish reproducibility of banding pattern of different DNA samples of turmeric. Both of the analysis were performed taking 10 randomly selected tissue culture derived (TP) and conventionally propagated (CP) plants (Fig. 1).

Field Establishment and morphological analysis of tissue culture derived plants

In vitro grown plantlets with well developed roots and shoots were transferred to pots containing soil, cow dung

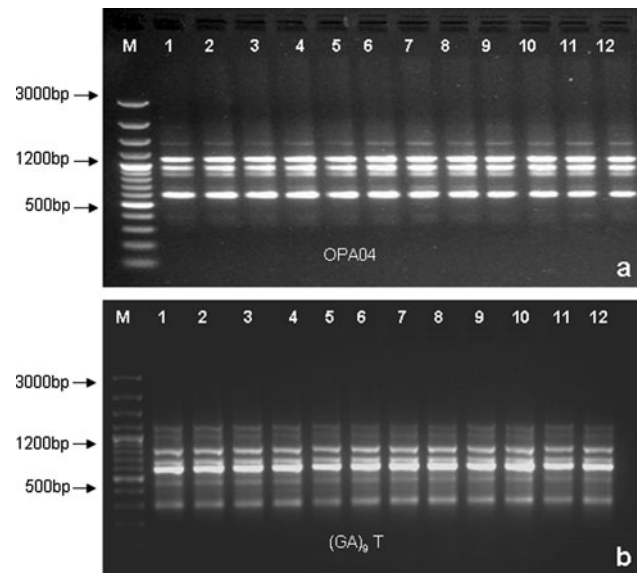


Fig. 1 **a** RAPD banding pattern in both micropropagated and field grown mother plants of *C. longa* (Lane 1 mother plant; Lane 2–12 micropropagated plants). **b** ISSR banding pattern in both micropropagated and field grown mother plants of *C. longa* (Lane 1, mother plant; Lane 2–12 micropropagated plants)

and sand mixture in 1:1:1 ratio after 90 days of growth in culture. These were kept in green house for acclimatization. After 30 days, pots were transferred to the normal field condition and grown to maturity. Tissue culture regenerated plants grown in the field were compared with conventionally propagated plants for various morphological biochemical and molecular characters. The characters scored were plant heights, number of tillers per plant, number of leaves, total rhizome weight. The morphological characters were recorded 6 months after transplanting.

Extraction and analysis of curcumin

Rhizomes of turmeric were collected from field before onset of dormancy (i.e. November) for extraction of curcumin. The rhizomes were cleaned thoroughly with water, cut into small pieces and air dried. The air dried rhizomes were powdered in mortar with a pestle, 0.1 g of powdered rhizome was taken in a flat bottom flask and 75 ml of acetone was added and refluxed for 4 h. The refluxed residue was cooled, filtered and washed with 100 ml of acetone. 10 ml of the filtrate was taken and diluted to 250 ml with acetone. The absorbance of the diluted sample and that of the standard curcumin (95% HPLC Purified, Charak) solution were measured at 420 nm by spectrophotometer (Thermo Scientific, UV 1) and curcumin percentage of the sample was estimated according to the ASTA method (ASTA Method No. 1.09 1997).

$$\begin{aligned} \text{Curcumin (\%)} &= (\text{absorbance of the sample}) \\ &\times (\text{dilution factor}) / (\text{factor derived from standard}) \\ &\times (\text{weight of the sample}) \end{aligned}$$

Extraction and analysis of oleoresin content

For oleoresin extraction, 100 g of turmeric rhizome (from which oil was already extracted) was taken. The rhizomes were transferred into extractor (soxhlet) by putting cotton in the lower bed. About 250 ml of the acetone was added in the flask and extraction apparatus was assembled. Extraction was continued until the color of the acetone become transparent. After extraction, evaporation of the acetone was done in water bath at 65°C and extract was transferred carefully into the beaker. For complete evaporation of acetone, extract was placed in hot air oven at 75°C for 1 h. The final extract found was oleoresin. The percentage of the oleoresin was calculated by ASTA analytical method (ASTA Method No. 1.06 1997).

$$\begin{aligned} \text{Oleoresin (\%)} &= (\text{weight of the residue}) / \\ &(\text{weight of the sample}) \times 100 \end{aligned}$$

Extraction and analysis of essential oil

Essential oil was extracted by hydro-distillation of fresh rhizomes and leaves of conventionally propagated and micropropagated turmeric plants in a Clevenger's apparatus following the method of Guenther (1972). The component identification was achieved by the GC–MS analysis using HP 6890 series GC (Hewlett-Packard, USA) coupled with mass selective detector (MSD), H P 5973 series (Hewlett-Packard, USA). Helium was used as carrier gas and the sample was injected in splitless mode in a column HP5 Phenyl methyl siloxane [25 µm (film thickness) × 320 µm (internal diameter) × 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: Initial temperature—60°C, Ramping rate—3°/min, Final temperature—243°C, Run time—61 min. For GC–MS evaluation 10 plants from each group (TP and CP) were randomly selected.

Result and discussion

In vitro sprouting and multiplication

The dormant axillary bud embedded in scales of unsprouted rhizome segments of conventionally propagated elite

clone of turmeric sprouted in vitro when cultured on MS medium supplemented with varying combinations and concentration of BA, Kn, IAA, and NAA. In vitro sprouting occurred within 7–10 days of explantation on media. Of all the combinations, medium containing 3 mg/l BA and 1 mg/l IAA was optimum for sprouting of axillary bud. 83% of axillary buds sprouted on on this media and multiplied further producing eight shoots approximately within 30 days of explantation. In all earlier reports of micropropagation of wild and cultivated turmeric direct shoot multiplication has been achieved using sprouted shoot buds of field grown rhizomes as explants, available only during planting season i.e., during onset of monsoon (Nadgauda et al. 1978; Balachandran et al. 1990; Salvi et al. 2001, 2002; Nayak and Naik 2006) excepting sole report of Panda et al. (2007) from our lab where axillary bud has been used as explant.

Thus round the year availability of dormant axillary buds for initiation of fresh turmeric culture rules out the problem of seasonal dependence that could arise due to use of active sprouting buds. Differential requirement of growth regulators for in vitro shoot multiplication reported in some elite cultivars of turmeric (Balachandran et al. 1990; Salvi et al. 2002; Nayak and Naik 2006) could be due to their genotypic differences. However BA has got marked effect in shoot multiplications in all such cases. Microshoots derived from in vitro sprouted axillary buds were rooted in the same media used for optimum shoot multiplication. Culture was maintained in vitro with regular sub culturing at 3 months interval. Multiplication rate remain unchanged even after 7 years of culture.

Micropropagated turmeric plants after 7 years of in vitro culture were transferred to polythene bags containing soil, cow dung, and sand mixture in 1:1:1 ratio during monsoon. These plantlets were acclimatized in the greenhouse for 1 month and about 96% plants survived under normal field condition. This procedure was followed in order to derive enough planting material of elite genotype 'Surama' with pre-formed rhizomes to speed up the growth of rhizome in field condition.

In vitro monitoring of genetic stability

In our study we adopted the method of periodic assessment of micropropagated plants of *C. longa* grown in vitro using cytophotometric, RAPD and ISSR analysis up to 7 years to detect the phase of induction of somaclonal variation at early or late phase of culture. The original explants (axillary bud) of turmeric were subjected to cytophotometric analysis before placement on to the media and revealed presence of unimodal peak corresponding to 2C value (7.6 pg) thereby confirming absence of variant nuclei. Cytophotometric analysis of 4C nuclear DNA content of

Table 1 4C nuclear DNA content, RAPD and ISSR profiling of in vitro conserved plantlets *Curcuma longa* at different culture period up to seven years

Period of culture (months)	No of regenerants analyzed	Range of mean 4C DNA content (pg)	Ploidy status of regenerants	No of bands amplified by RAPD primers	No of bands amplified by ISSR primers
Control (Surama plant from field)	10	7.63–7.68	Exclusively diploid (Ed)	650	640
1	12	7.65–7.66	Ed	780	768
6	15	7.63–7.68	Ed	975	960
12	17	7.65–7.70	Ed	1,105	1,088
18	17	7.62–7.65	Ed	1,105	1,088
24	15	7.63–7.65	Ed	975	960
30	22	7.65–7.68	Ed	1,430	1,408
36	21	7.61–7.64	Ed	1,365	1,344
42	20	7.65–7.68	Ed	1,300	1,280
48	23	7.63–7.66	Ed	1,495	1,472
54	20	7.64–7.67	Ed	1,300	1,280
60	18	7.63–7.69	Ed	1,170	1,152
66	30	7.63–7.68	Ed	1,950	1,920
72	35	7.62–7.65	Ed	2,275	2,240
78	38	7.63–7.66	Ed	2,470	2,432
84	50	7.65–7.68	Ed	3,250	3,200
Total	353			22,945	22,592

root tips of all regenerants analyzed over 7 years of culture period revealed diploidy in all showing range of variation from 7.63 to 7.70 pg, similar to the value obtained among source explants (Table 1). Cytophotometric analysis has also been used for the assessment of genetic stability among in vitro grown plantlets of *Ornithogalum thysooides* (Nayak and Sen 1991) *Ornithogallum umbellatum* (Nayak and Sen 1995) and *Zingiber officinale* (Mohanty et al. 2008, 2010). RAPD and ISSR markers were chosen because of simplicity and cost effectiveness and their efficiency in reliable monitoring of variability of DNA sequences among in vitro conserved plantlets (Zietkiewicz et al. 1994; Martins et al. 2004; Mohanty et al. 2008; Bhatia et al. 2009).

A total of 18 RAPD primers (OPA4, OPA7, OPA9, OPA18, OPC2, OPC5, OPC11, OPD3, OPD7, OPD8, OPD12, OPD18, OPD20, OPN4, OPN16, OPN18, OPAF5, OPAF14) out of 30 and 8 ISSR primers i.e., (GAC)5, (GTGC)4, (GACA)4, (AGG)6, (AGG)6, T(GA)9, (GTG)5 and (GGA)4 showing scorable and reproducible amplifications were utilized to analyse in vitro grown plantlets at different period of culture. A total of 22,945 bands (number of plants analyzed \times number of bands with all primers) generated by RAPD technique and 22,592 bands generated by ISSR primers over a period of 7 years in culture revealed genetic homogeneity showing monomorphic banding pattern throughout (Table 1). Size of monomorphic bands

ranged from 200 to 2,200 bp in case of RAPD analysis where as size of bands in case of ISSR analysis ranged from 200 to 2,900 bp. Intra clonal variations were also not observed among 10 normal field propagated source plants (control) using same RAPD and ISSR primers. These results has thus ensured the use of axillary bud derived culture for long term in vitro conservation of *C. longa* (cv Surama) to be used as disease free true to type stock plantlets. RAPD and ISSR based assessment of genetic stability of meristem culture derived micropropagated clones has been reported recently by Joshi and Dhawan in *Swertia chirayita* (2007) and Mohanty et al. (2008) in ginger.

Micropropagation through explants containing an organized meristem is generally regarded as having a lower risk of genetic instability (Shenoy and Vasil 1992). Nevertheless genetic stability is not guaranteed among meristem culture derived plantlets (Zucchi et al. 2002). Zucchi et al. (2002) studying somaclonal variation in sugarcane cultivars reported that DNA polymorphism detection in meristem culture derived somaclones could be attributed to pre existing polymorphism in source plant. In our present study absence of polymorphism in culture derived plantlets of turmeric could thus be due to absence of polymorphism in source plant. Genetic variation in meristem culture line could be dependent on the genotype used (Hammerschlag et al. 1987; Zucchi et al. 2002). We have recently reported genetic stability of micropropagated *C. longa* (cv Roma)

up to 26 months in culture using RAPD marker (Panda et al. 2007). However, the use of different genotypes and different explants of same genotype proved to have marked effect on the induction of somaclonal variation (Nayak and Sen 1998).

In the present study with cultivar Surama, length of culture period of 7 years with regular subculturing did not seem to affect their genetic integrity. This study in turmeric is in close agreement to that of Martins et al. (2004), Peredo et al. (2009) and Goto et al. (1998) showing genetic integrity among micropropagated plants of other species. However the correlation between the culture time length and the accumulation of somaclonal variation has been documented by several authors (Orton 1985; Hartmann et al. 1989).

This study showing relative stability of in vitro grown plants could be attributed to the use of explant devoid of pre-existing DNA variability and direct mode of regeneration. We have recently reported occurrence of somaclonal variation among callus derived regenerants (Nayak et al. 2008).

Evaluation of drug yielding potential of micropropagated turmeric in field

The genetic control of biochemical and morphological characteristics of plants to supply the market with crude drugs of uniform and stable quality is one of the major problems associated with commercial production of medicinal plant by tissue culture. The genetic instability of cultured tissue caused due to somaclonal variation has been shown to affect the chemical components of regenerated plants. Although this study involving cytophotometry and molecular analysis has not detected any variation among in vitro conserved plantlets, it cannot guarantee stability in their drug yielding potential due to the possibility that some changes occurring outside the priming site due to point mutation might have gone undetected. Therefore evaluation of drug yielding potential of micropropagated plants

was made through field analysis for commercial utilization of the protocol.

Field evaluation of CP and TP plants were compared for two generations (Table 2). Mean tiller number of TP plants which was significantly higher than CP plants in first generation could be due to the residual effect of cytokinin present in culture media. However the significant difference in mean tiller number was not marked among two groups of plants of 2nd generation in the field. Mean rhizome yield of the tissue culture derived plant was similar to the conventional rhizome propagated plants. Analysis of phytoconstituents such as essential oil content and curcumin content revealed that mean value was almost same in two generation studied when compared with that obtained in source plant (control). GC–MS based quality evaluation of leaf essential oil of micropropagated turmeric revealed presence of major compounds (alpha-phellandrene, 1,8-cinole, alpha-piene, liniole, geraniol) as found in their source plant. Similarly rhizome oil analysis revealed the presence of major compounds (Ar-tumerone, curlone, methyl-3-methyl-2-phenyl-2-butanol, clinasterol, alpha-phellandrene) as found in source plants. Rhizome oil of both CP and TP plants were dominated by ar-tumerone which comprise approximately 48% of peak area where as alpha-phellandrene was reported as major constituent of leaf oil comprising almost 58% of area. Alpha phellandrene and ar-tumerone have also been reported as major constituent of leaf and rhizome oil of turmeric by other authors (Leela et al. 2002; Kuanar et al. 2009; Behura and Srivastava 2004). No remarkable difference was observed in mean percentage of curcumin essential oil alpha phellandrene and ar-tumerone content between micropropagated and conventionally propagated turmeric in field thus showing absence of epigenetic changes. High uniformity in alkaloids of culture derived somaclones has also been reported in *Cymbopogon flexiosus* and *Aconitum carmichaeli* when compared with conventionally propagated clones in field (Nayak et al. 1996; Hatano et al. 1988).

Table 2 Field evaluation of different characteristics of micropropagated turmeric for 2 years

Characteristics	Control (CP plants)	1st year (TP plants)	2nd year (TP plants)
Plant height (cm)	63.4 ± 0.4	65.2 ± 0.8*	64.9 ± 0.7*
Tiller number	3.1 ± 0.2	3.7 ± 0.3*	3.6 ± 0.5*
Rhizome yield per plant (g)	81.3 ± 0.6	79.8 ± 0.5*	81.6 ± 0.3*
Essential oil content in leaves (%)	0.5 ± 0.2	0.52 ± 0.3	0.5 ± 0.3
Essential oil content in rhizome (%)	1.0 ± 0.2	1.0 ± 0.3	1.0 ± 0.3
Curcumin content (%)	5.5 ± 0.1	5.95 ± 0.2	6.1 ± 0.1
Oleoresin content (%)	12.1 ± 0.8	11.8 ± 0.7	12.3 ± 0.7
Alpha-phellandrene content of leaf oil (%)	57.8 ± 1.1	57.9 ± 0.8	57.8 ± 0.9
Ar-tumerone contentn of rhizome oil (%)	49.7 ± 0.5	49.5 ± 0.4	49.7 ± 0.6

* Significant at $P < 0.05$, rest are not significant

Thus the procedure outlined above suggests that axillary bud derived regeneration could be efficiently used for long term in vitro conservation of elite genotypes of turmeric (cv. Surama) with genetic and epigenetic stability for stable supply of drug to the market.

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