Assessment of Genetic Stability of Micropropagated *Curcuma caesia* through Cytophotometric and Molecular Analysis

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Summary Protocol has been standardized for micropropagation of medicinally important *Curcuma caesia* using sprouted bud explants from rhizome. Tissue culture raised plantlets of *C. caesia* could be conserved *in vitro* through subculturing at an interval of 4 months. The genetic stability of micropropagated plants was studied with an interval of 6 months up to 30 months in culture using cytophotometric, random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) analysis. Cytophotometric analysis of 151 plants revealed a unimodal distribution of the DNA content with a peak corresponding to 4C nuclear DNA (3.5 pg). RAPD and ISSR analysis revealed monomorphic bands showing the absence of polymorphism in all 73 regenerants analyzed, thus confirming the genetic uniformity among *in vitro* grown somaclones of *Curcuma caesia*.

Key words *Curcuma caesia*, Cytophotometric analysis, Genetic stability, Micropropagation, Molecular marker.

Curcuma caesia Roxb., commonly known as black turmeric is a wild species of the family Zingiberaceae. *C. caesia* is known especially in North East India and Bangladesh. It is rarely found in other parts of India, such as Madhya Pradesh, Jharkhand, Chattisgarh, and Orissa. Rhizomes of the plant are used for sprains and bruises, and are also employed as cosmetics (CSIR 1962). It is also used as a carminative, and for the treatment of headaches and rheumatic pains. The rhizomes are aromatic and yield an essential oil. The inner part of the rhizome is bluish-black in colour and emits a characteristic sweet smell due to the presence of essential oil. The oils possess antibacterial and antifungal properties (Rahman and Yusuf 1996) and are claimed to be useful in treating piles, leprosy, bronchitis, asthma, cancer, epilepsy, fever, wounds, impotency and migraines etc. A paste made from the rhizome is used to cure blood dysentery and as poultice in rheumatic pain. The leaves are used as a wrapping material and dry leaves are used as fuel by Bengalis in Bangladesh (Yusuf *et al.* 2002). North Indian tribes use black turmeric as a talisman to keep the evil spirits away. This plant is in great demand in Central India and due to indiscriminate exploitation without ensuring its regeneration and conservation, the plant has recently been categorized as an endangered species (Kumar 1998). This necessitates the application of the tissue culture technique for micropropagation and *in vitro* conservation of *C. caesia* and to ensure genetic stability for production of true-to-type plantlets.

The tissue culture technique is a powerful tool which can be employed as an alternative to the conventional method of vegetative propagation with the objective of rapid clonal multiplication of desired genotypes (Hussey 1986, Murashige and Skoog 1990). Periodic monitoring of the degree of

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genetic stability of *in vitro* conserved plants is of utmost importance for commercial utilization of the technique for large scale production of true-to-type plants of the desired genotype. The assessment of the genetic integrity of *in vitro* grown regenerants at regular intervals can significantly reduce or eliminate the chance of occurrence of somaclonal variations (Larkins and Scowcroft 1981) at the early or late phase of culture. Cytophotometric analysis is very useful for quick determination of the extent of the ploidy level of regenerated plants with a large number of cells (Nayak and Sen 1995). Of several molecular markers used for the assessment of genetic stability, RAPD and ISSR analysis are the simplest, cheapest and quickest method for determining the genetic fidelity of *in vitro* grown plants as reported in many species (Williams *et al.* 1990, Rout *et al.* 1998, Martins *et al.* 2004, Venkatachalam *et al.* 2007, Joshi and Dhawan 2007). Although micropropagation and *in vitro* conservation of *C. caesia* has been reported (Balachandran *et al.* 1990, Raju *et al.* 2005, Tyagi *et al.* 2004), there is no report regarding the extent of stability of the micropropagated clones.

We report rapid *in vitro* multiplication of *C. caesia* and *in vitro* monitoring of the genetic stability of micropropagated plants through cytophotometric estimation of the 4C nuclear DNA content RAPD and ISSR analysis at regular intervals. This will definitely meet the increasing demand of this endangered species with its conserved genetic integrity.

Materials and methods

Plant material

Rhizome of *C. caesia* was collected from a high altitude research station in Pottangi, Koraput, Orissa, and was grown in the medicinal plant garden of the Centre of Biotechnology, Bhubaneswar, Orissa (India). Rhizomes containing sprouted apical buds were used as explants and were thoroughly washed with water. The explants were kept under running water for 10–15 min and then dipped in liquid detergent (Extran, Merck) for 3–5 min. They were thoroughly washed with distilled water to remove the last drop of detergent. Surface sterilization was done by 0.1% mercuric chloride solution for 8–12 min depending upon the nature and size of the explants. After sterilization the explants containing buds were washed several times with sterile distilled water under aseptic conditions prior to inoculation.

In vitro multiplication

Explants were inoculated to the basal medium of Murashige and Skoog (MS) (1962) with varying combinations of benzyl adenine (1–5 mg/l), indole-3-acetic acid (0.5–2 mg/l), naphthalene acetic acid (0.5–2 mg/l) and kinetin (0.5–2 mg/l). fifteen replicas were used for each treatment. The MS media was gelled with 0.8% (m/v) agar (Himedia Laboratories Pvt. Ltd., Mumbai, India) and sterilized at 121°C and 1.06 kg/cm² pressure for 15 min. The pH of the media was adjusted to 5.73–5.74. After sterilization of the media, the explants were inoculated into the medium. The explants were inoculated under aseptic conditions and then kept in a culture room under white fluorescent light of 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 16 h photoperiod. The temperature of the culture room was maintained at 25 $\pm$ 2°C. Explants with well developed shoots and roots were subcultured at an interval of 3–4 months in culture. Each individual shoot was separated and inoculated to a fresh media for further growth. *In vitro* grown plantlets with well developed shoots and roots were transferred to pots containing sterilized soil. These were then kept in a greenhouse for acclimatization. After 1 month they were transferred to normal field conditions and grown to maturity.

Cytophotometric analysis

For cytophotometric analysis, explants' tissue was collected from a field-grown source plant

and the root tips of *in vitro* grown regenerants aseptically from culture tubes and fixed over night in propionic acid/ethanol (1 : 3). This treatment was followed by hydrolysis in 1 M 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 micro-spectrophotometer 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 4C nuclear DNA value of *Allium cepa* (67.1 pg) as standard (Van't Hof 1965).

Isolation of DNA

Fresh young leaves were collected and washed thoroughly with cold autoclaved distilled water, then blotted to dry. About 2 g of leaves were taken for extraction. The genomic DNA was isolated following the protocol of Doyle and Doyle (1990) with a little modification; insoluble polyvinylpyrrolidone was added to the leaf sample prior to grinding. The crude DNA was purified with RNase A (60 µg/ml of crude DNA solution) followed by washing thrice with phenol/chloroform/isoamyl alcohol (25 : 24 : 1 v/v/v) and subsequently with chloroform/isoamyl-alcohol (24 : 1 v/v). Quantification of DNA was done by 0.8% agarose gel electrophoresis of the samples along with a known amount of uncut lambda DNA (Bangalore Genei Pvt. Ltd, Bangalore, India) as standard. DNA samples were diluted to 25 ng/µl for RAPD-PCR analysis.

RAPD and ISSR analyses

For RAPD analysis a total of 30 random primers were used out of which 19 random decamer primers (Operon Tech, Alameda, USA) were selected. In the case of ISSR, out of 10 primers, 8 were selected. The RAPD analysis was performed as per the method of Williams *et al.* (1990) and for ISSR analysis the method of Zeitkiewicz *et al.* (1994) was followed. RAPD and ISSR amplifications were performed routinely using a PCR mixture (25 µl) containing 25 ng of genomic DNA as a template, 10×PCR buffer (Bangalore Genei, India), 200 µM dNTPs (Bangalore Genei, India), 0.5 unit (U) of Taq polymerase (Bangalore Genei, India), and 15 ng of RAPD primer or 40 ng of ISSR primer. The amplification was carried out in a thermal cycler (Gene Amp PCR system 9700, Applied Biosystems, CA, USA). In RAPD, PCR was performed at an initial temperature of 94°C for 5 min for complete denaturation. The second step consisted of 42 cycles having 3 ranges of temperature, *i.e.* at 92°C for 1 min for denaturation of template DNA, at 37°C for 1 min for primer annealing, at 72°C for 2 min for primer extension, followed by running the samples at 72°C for 7 min for complete polymerization. For ISSR the same temperature profile was followed, but the primer annealing temperature was set at 5°C lower than the melting temperature. The PCR products obtained from RAPD were analyzed in 1.5% agarose gel whereas the ISSR products were analyzed in 2% agarose gel stained with ethidium bromide (0.5 µg µl⁻¹). The size of the amplicons was estimated using 100 bp DNA ladder plus or DNA ladder mix (MBI Fermentas, Lithuania) and documented in the Gel Doc (Bio-Rad, USA).

Results

In vitro multiplication

Sprouted rhizome buds of *C. caesia* were cultured as explants (Fig. 1A) on MS media with various combinations of BA (1–5 mg/l), IAA (0.5–2 mg/l), NAA (0.5–2 mg/l) and Kinetin (0.5–2 mg/l). The percentage of explants forming shoots was maximum in media with BA (3 mg/l) and IAA (0.5 mg/l) *i.e.*, 92.8±0.3 (Table 1). A higher concentration of BA showed less response towards *in vitro* shoot initiation. Media with BA (1 mg/l) and IAA (0.5 mg/l) was optimum for *in vitro* shoot multiplication with 3.5±0.2 number of shoots and 1.1±0.2 number of roots (Fig. 1B, C). Media with BA and NAA were also effective for shoot initiation and multiplication. Media with

Fig. 1. (A) Sprouted bud explant of *C. caesia*. (B) Induction of multiple shoots in *C. caesia*. (C) *In vitro* multiplication in *C. caesia*. (D) Potted plant. (E) Micropropagated plants of *C. caesia* growing under field conditions.

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

Sl. No.	MS media with growth regulators (mg/l)				% of shoot initiation	No of shoots per explants (Mean $\pm$ SE)	No of roots per explants (Mean $\pm$ SE)
	BA	IAA	NAA	Kn			
1	1	—	—	—	30.6 $\pm$ 0.5	0.6 $\pm$ 0.2	0.5 $\pm$ 0.2
2	3	—	—	—	71.7 $\pm$ 0.3	1.2 $\pm$ 0.1	1.5 $\pm$ 0.2
3	5	—	—	—	61.5 $\pm$ 0.4	1.8 $\pm$ 0.3	1.4 $\pm$ 0.2
4	1	0.5	—	—	76.1 $\pm$ 0.5	3.5 $\pm$ 0.2	1.1 $\pm$ 0.2
5	3	0.5	—	—	92.8 $\pm$ 0.3	3.2 $\pm$ 0.2	2.1 $\pm$ 0.1
6	5	0.5	—	—	66.1 $\pm$ 0.6	2.1 $\pm$ 0.3	1.0 $\pm$ 0.3
7	1	—	0.5	—	71.7 $\pm$ 0.7	1.5 $\pm$ 0.2	1.5 $\pm$ 0.3
8	3	—	1	—	54.8 $\pm$ 0.5	1.2 $\pm$ 0.1	1.2 $\pm$ 0.1
9	1	—	—	0.5	43.4 $\pm$ 0.7	1.1 $\pm$ 0.1	2.5 $\pm$ 0.2
10	3	—	—	1	54.7 $\pm$ 0.6	1.1 $\pm$ 0.1	2.1 $\pm$ 0.2
11	1	0.5	0.5	—	38.7 $\pm$ 0.4	1.1 $\pm$ 0.3	2.1 $\pm$ 0.3
12	3	1	1	—	64.2 $\pm$ 0.6	1.4 $\pm$ 0.2	2.4 $\pm$ 0.2

The differences in mean values among the treatments are statistically significant ($p < 0.05$). Data represents the mean of 15 replicates for each treatment.

BA (1 mg/l) and NAA (0.5 mg/l) showed 71.7 $\pm$ 0.7 percentage of shoot initiation with 2.5 $\pm$ 0.2 number of shoot and 1.5 $\pm$ 0.2 number of roots. The number of shoots decreases with the increase in concentration of BA and NAA. Kinetin alone had no role in shoot multiplication.

Establishment of plants in soil

Plants with well developed shoots and roots (Fig. 1D) were then transferred to pots and grown

in green house for 1 month for acclimatization. After 1 month the acclimatized plants were transferred to the field and were established showing a survival rate of 90% (Fig. 1E).

Cytophotometric analysis

Cytophotometric analysis of the original explant was carried out before explantation which revealed a peak showing 2C value (1.7 pg). The cytophotometric analysis of the 4C nuclear DNA content of root tips from a total of 151 *in vitro* grown regenerants, carried out at 6 month, intervals up to 24 months in culture, revealed diploidy in all with a unimodal distribution with 1 distinct peak corresponding to a 4C value (3.5 pg). This is the same same as obtained in the source plant. The range of mean DNA content in root tips of *in vitro* conserved plantlets of different ages was almost similar to the range obtained in root tips of 10 control plants from the field (Table 2). The plantlets of *C. caesia* used for the cytophotometric study were labeled properly to be used for the molecular analysis with RAPD and ISSR markers.

Table 2. Nuclear DNA content of *in vitro* grown plantlets of *C. caesia* at different culture periods

Period of culture	No of plants Analyzed	Ploidy status of regenerants	Range of mean DNA Content (pg)
Control from field	10	Exclusively diploid	3.52–3.61
3 months	25	Exclusively diploid	3.54–3.58
6 months	32	Exclusively diploid	3.51–3.60
12 months	28	Exclusively diploid	3.52–3.62
24 months	31	Exclusively diploid	3.55–3.59
30 months	35	Exclusively diploid	3.53–3.61

RAPD and ISSR analyses

RAPD and ISSR analysis was done up to 30 months in a interval of 6 months. In *Curcuma caesia*, out of 25 RAPD primers 19 primers were selected and gave a total of 82 bands ranging from 320–3000 bp. The number of bands in all primers varied from 1–10 with an average of 4.3 bands per primer. All the bands were monomorphic, showing no polymorphism among 73 plants analyzed up to 24 months of culture (Fig. 2A). The number of

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

Table 3. RAPD and ISSR banding pattern of micropropagated and field-grown mother plants of *C. caesia*

	Primer	Sequence	Total bands	Range of amplicons [bp]
RAPD	OPA4	AATCGGGCTG	6	320–1500
	OPA7	GAAACGGGTG	2	600–2200
	OPA9	GGGTAACGCC	5	850–1750
	OPA18	AGGTGACCGT	3	450–900
	OPC2	GTGAGGCGTC	6	650–1300
	OPC5	GATGACCGCC	3	1031–1700
	OPC11	AAAGCTGCGG	10	450–1900
	OPD3	GTCGCCGTCA	2	800–1250
	OPD7	TTGGCACGGG	8	450–2000
	OPD8	GTGTGCCCCA	4	850–1700
	OPD12	CACCGTATCC	1	850
	OPD18	GAGAGCCAAC	5	450–1031
	OPD20	ACCCGGTCAC	6	600–3000
	OPN4	GACCGACCCA	3	500–1200
	OPN16	AAGCGACCTG	2	350–1031
	OPN18	GGTGAGGTCA	2	400–1031
	OPAF5	CCCGATCAGA	6	650–2200
	OPAF14	GGTGCGCACT	3	900–3000
	OPAF15	CACGAACCTC	5	550–2000
Total 82				
ISSR	SPS 1	(GAC)5	12	200–1200
	SPS 2	(GTGC)4	6	300–1050
	SPS 3	(GACA)4	8	475–1900
	SPS 4	(AGG)6	8	400–1031
	SPS 5	(GA)9T	5	350–975
	SPS 6	T(GA)9	7	500–1100
	SPS 7	(GTG)5	8	250–850
	SPS 8	(GGA)4	9	300–1500
Total 63				

monomorphic bands was highest, 10 in primer OPC11 (ranging from 450–1900 bp) and lowest, 1 in primer OPD12 (850 bp). A total of 5986 bands [(number of plantlets analyzed)×(number of bands with all primers)] were generated by the RAPD techniques (Table 3). Out of 10 ISSR primers, 8 primers formed a total of 63 bands(ranging from 200–1900 bp) which are all monomorphic in nature. The number of bands varied from 5–12 in all primers with an average of 7.8 bands per primer. A total of 4599 bands [(number of plantlets analyzed)×(number of bands with all primers)] were generated by the ISSR techniques (Table 3). The highest number of bands *i.e.* 12 in primer (GAC)5 (ranging from 200–1200 bp), and lowest number of bands, *i.e.* 5 in primer (GA)9T (350–975 bp), were amplified by ISSR analysis. Thus no variation was detected among *in vitro* conserved plantlets (Fig. 2B).

In the present study, 2 PCR based molecular markers, RAPD and ISSR, were used to show the genetic integrity in micropropagated *C. caesia* because of their cost effectiveness and simplicity. As there was no variation in the banding pattern both in RAPD and ISSR in all the regenerants analyzed in comparison with the mother plants, we conclude that the protocol developed could be used as a method for large scale propagation of true-to-type plantlets and conservation of *C. caesia in vitro*.

Discussion

In vitro multiplication of sprouted bud explants of *C. caesia* showed differential responses to MS media with various combinations of plant growth regulators. However BA had a marked effectiveness on multiplication. The role of BA in shoot multiplication has been reported in other Zingiberaceous species (Balachandran *et al.* 1990, Mohanty *et al.* 2008, Nayak 2000, Panda *et al.* 2007, Rout *et al.* 2002, Salvi *et al.* 2002). Development of roots occurred in the same media. This report is also in agreement with Tyagi *et al.* (2004). The plantlets could be conserved in MS media with BA 3 mg/l for more than 4 months whereas, according to Tyagi *et al.* (2004), kinetin was found to be optimum for the conservation of *C. caesia*. In our case, kinetin showed no such effect. This may be due to differences existing between *C. caesia* collected from different regions.

Somaclonal variation is a major problem associated with *in vitro* culture which arises as a direct consequence of *in vitro* culture of plant cell tissue and organs (Larkins and Scowcroft 1981). In our study we adopted the method of periodic assessment of micropropagated plants of *C. caesia* clones using cytophotometric, and RAPD and ISSR analysis to reduce the chance of induction of somaclonal variation at the early or late phase of culture.

Cytophotometric analysis of 4C nuclear DNA content of root tips of all regenerants at different culture periods revealed diploidy in all with a unimodal distribution of peak corresponding to a mean value of 4C nuclear DNA content (3.5 pg), the same as that of the source plant. The original explants were subjected to cytophotometric analysis before explantation onto the media and revealed the presence of unimodal peak corresponding to 2C value in each case, thereby confirming the absence of polysomaty.

Extensive work has been done to facilitate the understanding of mechanisms leading to somaclonal variations, which include chromosomal rearrangements, single gene mutations and lead to stable mutations. Hirochika (1993) first reported the activation of retrotransposons leading to somaclonal variations due to TC-induced stress in tobacco. The sub- and supra-optimal levels of plant growth substances, especially synthetic ones, have also been associated with somaclonal variation (Martin *et al.* 2006). Somatic embryogenesis is usually considered best next to axillary multiplication as there is stringent genetic control throughout the somatic embryo formation and the selection pressure against the abnormal types is considerably high (Leroy *et al.* 2000). Other methods of regeneration, such as organogenic differentiation, are more prone to somatic variations. Shenoy and Vasil (1992) reported that micropopagation through explants containing organized meristem is generally associated with low risk of genetic instability. Hence, it becomes imperative to regularly check the genetic purity of the micropopagated plants in order to produce clonally uniform progeny while using different techniques of micropopagation.

In the present study, the length of culture period (for 30 months) did not seem to affect their genetic stability. Some authors have reported that the time in *in vitro* culture could promote somaclonal variation (Hartmann *et al.* 1989, Nayak and Sen 1991), whereas Gould (1986) has reported that culture time does not seem to be the only parameter affecting genetic stability. Genetic variation in a culture line could be affected more by a genotype than by the period in culture (Hammerschlag *et al.* 1987, Nayak and Sen 1998, Vendrame *et al.* 1999).

In the present study, RAPD and ISSR markers were chosen because of their simplicity and cost-effectiveness. The use of 2 types of markers which amplify different regions of the genome allow better analysis of genetic stability/variation of the plantlets (Martins *et al.* 2004, Venkatachalam *et al.* 2007). In the present study, RAPD and ISSR analysis of *in vitro* conserved *C. caesia* showed a profile similar to that of the control, indicating that no genetic variation had occurred *in vitro*. This confirmed the genetic integrity by RAPD and ISSR analysis that had been reported earlier in many species (Bhatia *et al.* 2009, Joshi and Dhawan 2007, Mohanty *et al.* 2008, Panda *et al.* 2007, Rout *et al.* 2002, Venkatachalam *et al.* 2007, Salvi *et al.* 2002, Martins *et al.*

2004). Our study, in close agreement with Zuchi *et al.* (2002), reveals that the absence of polymorphism in micropropagated *C. caesia* could be due to the absence of polymorphism in source plants.

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