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Genetic Stability Assessment of Micropropagated Mango Ginger (*Curcuma amada* Roxb.) Through RAPD and ISSR Markers

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ABSTRACT

Curcuma amada Roxb., popularly known as mango ginger is an important spice and medicinal plant of family Zingiberaceae. In this attempt, amplified polymorphic DNA (RAPD) and Inter Simple Sequence repeats (ISSR) markers were used to determine the genetic stability of micropropagated *Curcuma amada*. Fifty regenerants were analyzed each at an interval of six months up to two years in culture. Out of 25 RAPD and 10 ISSR primers screened, 19 RAPD primers and 8 ISSR primers gave 3100 and 3300 bands, respectively. RAPD and ISSR analysis revealed monomorphic bands showing the absence of polymorphism in all regenerants analyzed, confirming their genetic uniformity. These results suggest that the micropropagation protocol developed by us for rapid *in vitro* multiplication is appropriate and applicable for clonal mass propagation of *Curcuma amada*.

Key words: *Curcuma amada*, rapid amplified polymorphic DNA, inter simple sequence repeats, genetic stability, micropropagation

INTRODUCTION

Curcuma amada (mango ginger) an under exploited, important medicinal plant is cultivated for its aromatic rhizome. The rhizome is carminative, stomachic and applied over contusions and sprains (Nayak, 2002). It is also used in the treatment of bronchitis, asthma, cough itching and skin diseases. The root is diuretic, emollient, expectorant, astringent, antipyretic, appetizer and is used in mouth, ear diseases, lumbago and diarrhoea (Kirtikar and Basu, 1984). Due to its mango like aroma, it is used in pickles and culinary preparations. Two novel bioactive compounds were isolated and characterized from mango ginger rhizome having antimicrobial, antioxidant, platelet aggregation inhibitory activity and antitubercular activity (Policegoudra *et al*., 2010; Singh *et al*., 2010). *C. amada* is a vegetatively propagated plant with very low multiplication rate. Lack of seed set in this plant discourages conventional breeding efforts. In addition, its susceptibility to various rhizome rot diseases cause huge loss during each year. Hence, large amount of fresh planting materials are required each year for plantation (Barthakur and Bordoloi, 1992). These problems necessitate an alternative technique for the production of true-to-type planting material for *C. amada*. Nevertheless, *in vitro* culture has the potential to increase the multiplication rate of elite genotypes and to produce improved cultivars when combined with other tools of biotechnology.

In vitro clonal multiplication of other *Curcuma* species through rhizome buds has been reported by many authors (Nadgauda *et al.*, 1978; Balachandran *et al.*, 1990; Salvi *et al.*, 2002). Available reports are however limited only to micropropagation of *C. amada* (Barthakur and Bordoloi, 1992), regeneration from leaf sheath callus and microrhizome production (Nayak, 2002). There is no such report describing clonal fidelity of micropropagated plants using molecular markers. Somaclonal variation is the major problem associated with *in vitro* culture among sub-clones of one parental line, arising as a direct consequence of *in vitro* culture of plant cells, tissue or organs (Gould, 1986; Larkin and Scowcroft, 1981). Periodic monitoring of genetic stability of *in vitro* conserved plants is important for commercial utilization of true-to-type plants of the desired genotype. Hence, 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 variation (Larkin and Scowcroft, 1981; Rani *et al.*, 1995) at the early or late phase of culture. Molecular techniques are at present powerful and valuable tools used in analysis of genetic fidelity of *in vitro* grown plants and are the subject of many publication and reviews. Of the various molecular markers used, Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) analysis are the simplest and quickest tools for genetic stability assessment of *in vitro* grown plants as reported in many species (Bhatia *et al.*, 2009; Modgil *et al.*, 2005; Mohanty *et al.*, 2010).

The aim of the present study was to report micropropagation, *in vitro* conservation and genetic stability assessment of *in vitro* conserved plantlets of medicinally important *Curcuma amada*. For this study, one clone has been derived and tested by two molecular markers, RAPD (Williams *et al.*, 1990) and ISSR (Zeitkiewicz *et al.*, 1994).

MATERIAL AND METHODS

Plant material: Healthy and sprouted rhizomes of *C. amada*, collected from High altitude research station, Pottangi, Koraput, Orissa, were washed properly in tap water followed by a liquid detergent (Extran, Merck, Mumbai, India) for 10 min and wash with sterilized water. These were then surface sterilized with 0.1% (w/v) mercuric chloride for 11-12 min. After rinsing with sterile distilled water three times, explants were used for culture initiation. Approximate size of the explants was 10-12 mm in length.

***In vitro* multiplication and culture conditions:** Explants were inoculated in MS medium (Murashige and Skoog, 1962) containing 30 g L^{-1} sucrose and different concentration of BA (0.5-5 m g L^{-1}), IAA (0.5-1 m g L^{-1}) and Kinetin (1-3 m g L^{-1}). Explants were first inoculated in the shoot induction media and then subcultured to another media for shoot multiplication following the protocol of Mohanty *et al.* (2010).

Field transfer: After acclimatization in the greenhouse, the plants were transferred to normal atmospheric conditions and were grown to maturity. All the experiments were repeated three times with a minimum of ten replicates.

DNA extraction: Healthy and young leaves of *C. amada*, were taken both from *in vitro* and *ex vitro* grown mother plants. Leaf samples were taken in every six months interval up to two years for RAPD and ISSR analysis. DNA extraction was done by following Doyle and Doyle (1987) method.

RAPD and ISSR analysis: RAPD and ISSR analysis was done up to 2 years with an interval of six months. For RAPD analysis, 30 random primers were used, out of which 19 random decamer primers (Operon Tech, Almeda, USA) were selected as responded well. In 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.

Statistical analysis: Data were subjected to analysis of variance for a factorial experiment. Critical Differences (CD) were calculated to determine the statistical significance of different treatment means.

RESULT AND DISCUSSION

***In vitro* multiplication and conservation:** In *C. amada*, sprouted buds of rhizome were taken as explants (Fig. 1a) for the initiation of *in vitro* culture. Various combinations of BA (1-5 mg L^{-1}), IAA (0.5-2 mg L^{-1}) and kinetin (0.1-1 mg L^{-1}) were tried for shoot initiation and multiplication (Table 1). MS media with 3 mg L^{-1} BA showed maximum percentage of shoot initiation i.e.,

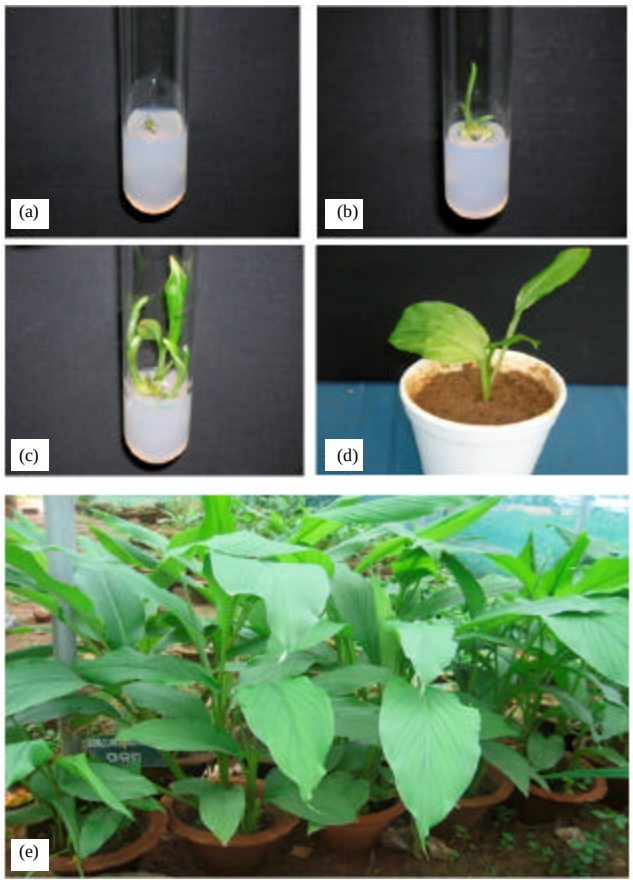

Fig. 1(a-e): (a) *Curcuma amada* explants, (b, c) Shoot and root multiplication, (d) Potted plant and (e) Tissue cultured plants in greenhouse

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

MS media with growth regulators (mg L^{-1})					
BA	IAA	Kn	Shoot initiation (%) (Mean±SE)	Shoots per explants (Mean±SE)	Roots per explants (Mean±SE)
1	-	-	44.8±0.8^{e}	0.4±0.2^{a}	0.4±0.3^{a}
3	-	-	87.0±0.5^{e}	0.6±0.2^{a}	2.4±0.2^{d}
5	-	-	73.0±0.5^{d}	1.2±0.2^{a}	1.4±0.2^{a}
1	0.5	-	52.2±0.6^{c}	1.4±0.2^{b}	1.4±0.3^{a}
2	0.5	-	82.8±0.5^{d}	3.8±0.2^{h}	2.4±0.2^{d}
5	0.5	-	41.6±0.5^{b}	1.6±0.3^{b}	1.4±0.4^{a}
1	-	0.1	37.0±0.7^{b}	1.0±0.0^{a}	0.8±0.3^{a}
3	-	0.5	30.8±0.4^{b}	1.6±0.2^{b}	1.2±0.2^{a}
1	0.5	0.5	38.0±0.3^{b}	0.6±0.3^{a}	1.0±0.3^{a}
3	1	0.5	28.6±0.6^{b}	2.8±0.2^{e}	0.8±0.2^{a}
5	1	1	21.0±0.8^{a}	1.0±0^{a}	2.8±0.2^{e}

Mean having same letter in a column were not significantly different at $p<0.005$ level, Data represent the mean of 15 replicates for each treatment, Kn: Kinetin, BA: Butyric acid, IAA: Indde acetic acid, Kn: Kinetin

87.0±0.5 after 20-25 days of culture (Fig. 1b). When *in vitro* grown shoots were transferred to media with 2 mg L^{-1} BA and 0.5 mg L^{-1} IAA, 3.8±0.2 number of shoots was formed with 2.4±0.2 numbers of roots (Fig. 1c, d). At low concentration of BA fewer shoots were obtained. This report is in agreement with Prakash *et al.* (2004) who reported maximum number of shoots in media containing MS and BAP. BA with NAA showed negligible response in both shoot initiation and multiplication which is in contrast to the report of Barthakur and Bordoloi (1992). Shoots and roots developed in the same media. *In vitro* conservation could be done by keeping the plants on the half MS media with 2 mg L^{-1} BA, 30 g L^{-1} sucrose and 10 g L^{-1} mannitol by subculturing at an interval of 10 months. *In vitro* grown cultures were then transferred to multiplication media and 90% of the plants resumed normal growth. Cultured plants were successfully established in field after 30 days of acclimatization in green house (Fig. 1e).

RAPD and ISSR analysis: In *C. amada*, 19 primers were selected, out of 30 RAPD primers tried on the basis of good resolution and reproducibility. 62 scorable bands were formed ranging from 320-3000 bp (Table 2). Average number of bands per primer was 3.2, highest number of band was 6 in primer OPC5 (ranging from 1031-2500 bp) and lowest number of bands i.e., 1 in OPA9 (1400 bp) and OPD12 (1031 bp). 3100 number of bands produced [(number of plantlets analyzed) x (number of bands with all primers)] by RAPD techniques were all monomorphic (Fig. 2a) without having any polymorphism in all 50 plants analyzed. ISSR analysis in *C. amada* with 10 primers was done up to 2 years with 6 months interval. Out of ten primers, eight were selected due to their clarity. 66 bands were produced by 8 ISSR primers ranging from 250-2900 bp, with an average of 8.3 bands per primer. All bands are monomorphic in nature (Fig. 2b). Highest number of monomorphic band was found to be 14 in primer (GGA)4 (ranging from 300-1500 bp) and lowest of 4 in primer (GTGC)4 and (GA)9T (ranging from 500-1200 bp). A total of 3300 bands [(number of plantlets analyzed)×(number of bands with all primers)] were generated by the ISSR techniques (Table 3). All 50 *in vitro* conserved plantlets analyzed were true-to-type showing no variation through out the period of study.

Numerous studies on detection of somaclonal variations have been done using PCR-based techniques such as RAPD, ISSR, SSR and AFLP and RAPD being one of the most used. In our

Table 2: RAPD banding pattern of micropropagated and field-grown mother plants of *C. amada*

Primer	Sequence	Total bands	Range of amplicons (bp)
OPA4	AATCGGGCTG	5	600-1475
OPA7	GAAACGGGTG	5	500-2200
OPA9	GGGTAACGCC	1	1400
OPA18	AGGTGACCGT	3	575-1075
OPC2	GTGAGGCGTC	2	700-1200
OPC5	GATGACCGCC	6	1031-2500
OPC11	AAAGCTGCGG	3	600-1250
OPD3	GTCGCCGTCA	3	1031-1400
OPD7	TTGGCACGGG	5	450-1250
OPD8	GTGTGCCCCA	4	850-2000
OPD12	CACCGTATCC	1	1031
OPD18	GAGAGCCAAC	4	700-1500
OPD20	ACCCGGTCAC	4	600-1500
OPN4	GACCGACCCA	3	500-1200
OPN16	AAGCGACCTG	3	320-1200
OPN18	GGTGAGGTCA	2	450-1050
OPAF5	CCCGATCAGA	3	700->3000
OPAF14	GGTGCGCACT	3	700-1031
OPAF15	CACGAACCTC	2	1031-1500
Total		62	

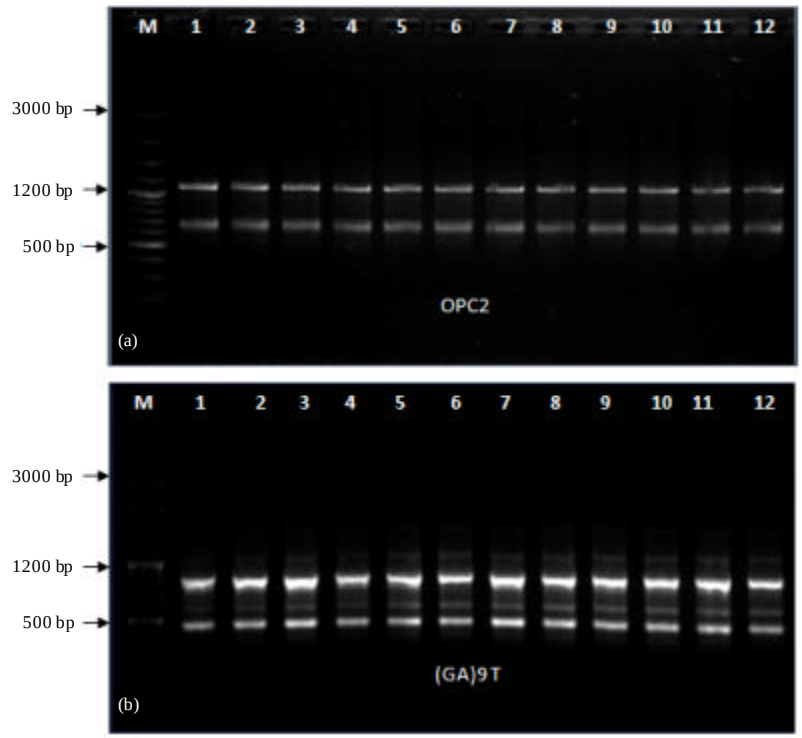

Fig. 2(a-b): (a, b) RAPD and ISSR banding pattern in both micropropagated and field grown mother plants of *C. amada* (Lane 1: Mother plant; Lane 2-12: Micropropagated plants)

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

Primer	Sequence	Total bands	Range of amplicons (bp)
SPS 1	(GAC)5	11	350-1200
SPS 2	(GTGC)4	4	300-1050
SPS 3	(GACA)4	8	600-2900
SPS 4	(AGG)6	9	325-1031
SPS 5	(GA)9T	4	500-1200
SPS 6	T(GA)9	9	400-1100
SPS 7	(GTG)5	7	250-850
SPS 8	(GGA)4	14	300-1500
Total		66	

study two PCR based molecular markers i.e., RAPD and ISSR were used to show the genetic integrity in micropropagated *C. amada* because of their cost effectiveness and simplicity. The use of two 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). Palombi and Damiano (2002) also suggested the use of more than one DNA amplification technique as advantageous in evaluating somaclonal variation. In *C. amada,* the duration plantlets were kept in culture (two years) did not seem to affect their genetic integrity. Martins *et al*. (2004), Angel *et al*. (1996) and Mohanty *et al*. (2010) also found no variation in regenerants kept in *in vitro* culture for more than 2 years. Some authors however have reported that the time in *in vitro* culture could promote somaclonal variation (Hartmann *et al*., 1989; Orton, 1985). According to Gould (1986) culture time does not seem to be the only parameter affecting genetic stability. Vendrame *et al*. (1999) reported that genetic variation in a culture line could be affected more by the genotype than by the period in culture. Genotype and the nature of the explants could influence the phenotypic stability of the plant obtained (Hammerschlag *et al*., 1987).

Mode of regeneration also affects the genetic stability of micropropagated plants. Micropropagation through explants containing an organized meristem is generally regarded as having a lower risk of genetic instability (Shenoy and Vasil, 1992). Our study, in close agreement to Shenoy and Vasil (1992) reveals that the relative stability of micropropagated *C. amada*, could be due to the direct mode of plant regeneration through multiplication of sprouted bud of rhizome.

Among species of Zingiberaceae, molecular marker based assessment of genetic stability of micropropagated plantlets are limited to cultivated species of *C. longa* and *Z. officinale* using RAPD analysis only (Mohanty *et al*., 2008; Panda *et al*., 2007) lacking any report on stability analysis using ISSR markers. In the present study RAPD and ISSR analysis of *in vitro* conserved *C. amada* showed a profile similar to that of the control indicating that no genetic variation had occurred *in vitro,* confirming their genetic integrity. RAPD and ISSR analysis of *in vitro* grown plants has been reported earlier in many species (Bhatia *et al*., 2009; Joshi and Dhawan, 2007; Martins *et al*., 2004; Mohanty *et al*., 2008; Panda *et al*., 2007; Rout and Das, 2002; Salvi *et al*., 2002; Venkatachalam *et al*., 2007).

CONCLUSION

An efficient protocol on micropropagation of *C. amada* has been developed for the first time with genetic integrity. Our results demonstrate that RAPD and ISSR analysis can be applied to asses the genetic integrity of *in vitro* conserved plantlets of *C. amada,* on a large scale, there by facilitating the crop improvement programme in *Curcuma* species.

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