Full Length Research Paper

Evaluation of genetic fidelity of *in vitro* propagated shampoo ginger (*Zingiber zerumbet* (L.) Smith) using DNA based markers

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Drug yielding potential of shampoo ginger (*Zingiber zerumbet* (L.) Smith) is due to the presence of important phytoconstituent such as Zerumbone, which is currently being explored for its effects on cancer and HIV. Slow multiplication rate, high susceptibility to rhizome rot and leaf spot disease restricted the availability of the wild gingers. Thus, a protocol has been developed for *in vitro* propagation of Z. *zerumbet* using sprouted bud explants from rhizome. The explants in MS media with 4 mg/L benzyl adenine, 1 mg/L indole-3-acetic acid showed highest percentage of response, that is, 93.8%. The aseptic shoots of Z. *zerumbet* were formed with 5 multiple shoots on MS media with 3 mg/L benzyl adenine and 1 mg/L indole-3-acetic acid and 100 mg/L adenine sulfate. *In vitro* grown plantlets of Z. *zerumbet* could be conserved in MS media containing 0.5 mg dm^{-3} of benzyladenine and 60 mg/L of sucrose subcultured at an interval of eight months. Survival of *in vitro* conserved plants on multiplication media was 85%. Genetic stability of micropropagated clones were periodically evaluated at an interval of 6 months up to 30 months in culture using random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) analysis and genetic uniformity in all regenerants was confirmed.

Key words: Shampoo ginger, micropropagation, genetic fidelity, DNA markers.

INTRODUCTION

Zingiber zerumbet (L.) Smith, a member of the family *Zingiberaceae*, well known as "shampoo ginger" is distributed in Bangladesh, India, Malaysia, Nepal and Sri Lanka. It has been used traditionally for the treatment of stomach ache, toothache, fever, sprain and indigestion (Huang et al., 2005). The species also possesses analgesic, anti-pyretic and anti-inflammatory properties (Somchit et al., 2005). The volatile oil of the rhizomes has been shown to contain zerumbone, humulene and camprene The most studied chemical compound is the sesquiterpene zerumbone, which is reported to be a

potent inhibitor of a tumor promoter. Zerumbone has potent anti-inflammatory and chemo-preventive qualities. In spite of their great economical potential, the wild species of Zingiberaceae have received much less attention from biotechnologists for their conservation, characterization and genetic improvement following different biotechnological approaches. Slow multiplication rate, high susceptibility to rhizome rot and leaf spot disease restricted the availability of the wild gingers. These problems can be alleviated through the application of a tissue culture technique, an efficient, long-recognized tool for rapid multiplication of true-to-type genotypes (Tyagi et al., 2004; Mohanty et al., 2008). Periodic monitoring of the degree of 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

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Abbreviations: ADS, Adenine sulphate; **BA,** 6-benzyladenine; **GA,** gibberlic acid; **IAA,** indole acetic acid; **MS,** Murashige and Skoog; **NAA,** α - naphthalene acetic acid.

or eliminate the chance of occurrence of somaclonal variations (Larkins and Scowcroft, 1981) at the early or late phase of culture. Of several molecular markers used for the assessment of genetic stability, random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (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; Martins et al., 2004; Venkatachalam et al., 2007; Joshi and Dhawan, 2007). However, papers available so far on the micropropagation of *Z. zerumbet* (Tyagi et al., 2006, Shinija et al., 2009) exclude any information on molecular marker-based assessment of genetic stability.

We report rapid *in vitro* multiplication of *Z. zerumbet* and *in vitro* monitoring of the genetic stability of micropropagated plants through RAPD and ISSR analysis at regular intervals. This will definitely meet the increasing demand of this wild ginger with its conserved genetic integrity.

MATERIALS AND METHODS

Rhizomes of *Z. zerumbet* collected from Kalimpong, West Bengal, India were planted in soil to initiate sprouting. Pieces of rhizomes containing sprouts were washed thoroughly with water followed by a liquid detergent (Extran, Merck, Mumbai, India) for 10 min and again with sterilized water. These were then surface sterilized with 0.1 % (m/v) mercuric chloride for 11to 12 min. After rinsing with sterile distilled water three times, explants (10 to 12 mm) were inoculated to shoot induction Murashige and Skoog (1962) medium containing 30 g/L sucrose and different concentration of 6-benzyladenine (BA; 0.5 to 6.0 mg/L), indole-3-acetic acid (IAA; 0.5 to 2.0 mg/L), naphthaleneacetic acid (NAA; 0.5 to 2.0 mg/L), gibberlic acid (GA; 0.5 to 2.0 mg/L) and adenine sulphate (ADS; 80 to 100 mg/L). The pH of the medium was adjusted to 5.7 before adding agar to it. It was then autoclaved at 121°C and 104 kPa for 20 min. The culture was maintained at temperature of 25 ± 1°C, 16-h photoperiod (irradiance of 55 μmol m^{-2}s^{-1}. The percentage of response of those explants to different hormonal combinations was observed at different times. Culture conditions were similar to those used at the time of initial inoculation. *In vitro* grown plantlets having well-developed shoots and roots were transferred to pots containing soil, cow dung and sand mixture in 1:1:1 ratio after 90 days of growth in culture. These were then kept in a greenhouse for acclimatization. After 30 days, they were transferred to the normal field conditions and grown to maturity. All the experiments were repeated three times with a minimum of ten replicates. Micropropagated plants and conventionally propagated plants were compared for various morphological, biochemical and molecular characters. After 6 months of transplantation, the morphological characters such as plant length, leaf biomass, leaf number, rhizome weight and percentage of rhizome oil were recorded.

Healthy and young leaves of *Z. zerumbet* 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 (1990) method. The crude DNA was purified by adding Rnase A (60 μg for 1 cm^3 of crude DNA solution). Quantification of purified DNA was accomplished by analyzing the purified DNA in 0.8% agarose gel alongside uncut lambda DNA as standard. Purified DNA samples were then diluted in T10 E1 buffer to 25 μg cm^{-3} for PCR amplification. For RAPD analysis, a total of 30 random primers were

used out of which 17 random decamer primers (*Operon Technologies*, Almeda, USA) were selected. In case of ISSR; out of 10 primers 8 were selected. The RAPD analysis was performed according to Williams et al. (1990) and ISSR analysis according to Zeitkiewicz et al. (1994). RAPD and ISSR amplifications were performed routinely using PCR mixture (0.025 cm^3) containing 25 ng of genomic DNA as template, 10× PCR buffer (*Bangalore Genei*, Bangalore, India), 200 μM dNTPs (*Bangalore Genei*), 0.5 U of Taq polymerase (*Bangalore Genei*), 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, Carlsbad, CA, USA). In RAPD, PCR was performed at initial temperature of 94ºC for 5 min for complete denaturation. The second step consisted of 42 cycles having three ranges of temperature, that is, 92°C for 1 min for denaturation of template DNA, 37°C for 1 min for primer annealing, 72°C for 2 min for primer extension, followed by running the samples at 72ºC 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 mg cm^{-3}). The size of the amplicons were estimated using 100 bp DNA ladder plus or DNA ladder mix (*MBI Fermentas*, Vilnius, Lithuania) and documented in the *Gel Doc* (*Bio-Rad*, Hercules, USA).

RESULTS AND DISCUSSION

Sprouted buds of Z. *zerumbet* were taken as explants for *in vitro* induction of shoots. The explants in MS media with BA (4 mg/L) IAA (mg/L) showed highest percentage of response which is 93.8. Further increase in BA concentration decreases the response. The multiplication of shoot buds occurred in all media mentioned, but the rate of multiplication was relatively more in the media containing BA (3 mg/L) and IAA (1 mg/L) and Ads (100 mg/L). In this media, the aseptic shoots of Z. *zerumbet* were formed with 5 multiple shoots and 2.8 number of roots (Table 1). The role of BA in shoot multiplication has been reported in other Zingiberaceous species (*e.g.* Balachandran et al., 1990; Panda et al., 2007; Mohanty et al., 2008, 2011). 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 1 mg/L for more than 8 months. *In vitro* grown plantlets were then transferred to pots containing sand and soil in the ratio 1:1. Plants were hardened for seven days then were transferred to green house and then grown in normal field condition. About 90% of plants were survived and grown to maturity. Tissue culture-derived plants were assessed for their stability by comparing various morphological characters with those of conventionally grown Z. *zerumbet*. The result of the present study reveals no significant difference in mean value of any of the characters studied between the two groups of plants (Table 2). Our result is in close agreement with the findings of Nayak et al. (1996), who observed high uniformity in essential oil content of culture-derived clonal plants of *Cymbopogon flexuosus* Nees. (Wats) compared to the field-cultivated clones.

Table 1. *In vitro* shoot multiplication of *Z. zerumbet* on MS medium fortified with different growth regulators (Means ± SE, *n* = 15, *p* < 0.01).

S/N	MS media with growth regulators [mg dm⁻³]					Shoot initiation (%)	No. of shoots (explants⁻¹)	No. of roots (explants⁻¹)
	BA	IAA	NAA	GA	Ads			
1	1	-	-	-	-	33.8 ± 0.7	0.6 ± 0.2	0.6 ± 0.2
2	3	-	-	-	-	36.2 ± 0.6	1.2 ± 0.2	0.8 ± 0.2
3	2	0.5	-	-	-	75.0 ± 0.5	1.2 ± 0.3	1.4 ± 0.2
4	4	1	-	-	-	93.8 ± 0.7	0.8 ± 0.3	2.2 ± 0.2
5	6	1	-	-	-	54.6 ± 0.6	0.8 ± 0.2	0.8 ± 0.2
6	1	0.5	-	-	80	63.6 ± 0.8	2.8 ± 0.3	2.4 ± 0.2
7	3	1	-	-	100	75.2 ± 0.8	5.0 ± 0.3	2.8 ± 0.2
8	5	1	-	-	100	47.2 ± 0.5	2.4 ± 0.2	2.4 ± 0.2
9	2	0.5	-	0.5	-	56.4 ± 0.8	3.2 ± 0.3	1.4 ± 0.2
10	5	1	-	-	100	45.8 ± 0.5	2.4 ± 0.2	0.8 ± 0.2
11	2	-	0.5	0.5	-	43.2 ± 0.7	1.4 ± 0.2	0.8 ± 0.2
12	5	-	1	1	-	41.6 ± 0.8	1.2 ± 0.2	1.2 ± 0.2
13	2	-	0.5	-	100	74.6 ± 0.8	2.6 ± 0.2	2.0 ± 0.3
14	5	-	1	-	100	58.0 ± 0.6	2.4 ± 0.2	1.6 ± 0.4

Table 2. Comparison of different morphological characteristics between conventionally propagated plants and culture generated plants of *Z. zerumbet.*

Character	Conventionally propagated plants (Mean ± SD)	Micropropagated plants (Mean ± SD)
Plant length (cm)	118.08 ± 2.06	20.26 ± 0.49
Leaf biomass(gm)/plant	156.70 ± 6.20	187.81 ± 0.42
No. of leaves/plant	78.71 ± 2.99	86.4 ± 1.39
No. of tillers/plant	6.3 ± 0.53	6.8 ± 0.38
Rhizome yield (gm)/plant	153.4 ± 1.36	165.1 ± 0.68

Hatano et al. (1988) also observed a high uniformity in the alkaloid content of culture-derived somaclones of *Aconitum carmicheli* (Debeaux) compared to the field-cultivated clones.

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 Z. *zerumbet* clones using RAPD and ISSR analysis to reduce the chance of induction of somaclonal variation at the early or late phase of culture. RAPD analysis of micropropagated *Z. zerumbet* was carried out with 25 primers out of which 17 primers gave scorable results (Table 3).

RAPD analysis was carried out at an interval of 6 months up to two years. A total of 61 regenerants were analyzed taking a minimum of 10 at a time. RAPD analysis with 17 primers gave rise to a total of 74 scorable bands, ranging from 350 to >3000 bp. The number of bands for each primers was varied from 2 to 8, with an average of 4.35 bands per primer. 4514 number of bands [(number of plantlets analyzed) × (number of bands with all primers)], generated by the RAPD techniques, showed monomorphic patterns across all 61 plantlets analyzed.

Initially, a total of 10 ISSR primers were screened to assess the genetic integrity of the tissue cultured plants of *Z.zerumbet* out of which 8 primers were selected due to their clarity. ISSR analysis gave rise to 47 bands ranging from 325 to 1700 bp with an average of 5.8 bands per primer, highest in (GTGC)4 ranging from 550 to 1031 bp and lowest in T(GA) 9 ranging from 475 to 850 (Table 3). 2867 bands [(number of plantlets analyzed) × (number of bands with all primers)] were generated by the ISSR techniques. All the bands were monomorphic.

The use of two types of markers, which amplify different regions of the genome, allows for better analysis of genetic stability/variation among the somaclones (Martins et al., 2004; Venkatachalam et al., 2007). RAPD profiling of *in vitro*-grown micropropagated plants has been reported in many other plant species (Rani et al., 1995; Rout and Das, 2002; Martins et al., 2004;

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

RAPD primer	Sequence	Total band	Range of amplicons [bp]
OPA4	AATCGGGCTG	7	825 - 2000
OPA7	GAAACGGGTG	3	500 - 1031
OPA9	GGGTAACGCC	2	700 - 1600
OPA18	AGGTGACCGT	6	600 - 2250
OPC2	GTGAGGCGTC	5	700 - 1600
OPC5	GATGACCGCC	2	1250 - 2000
OPC11	AAAGCTGCGG	3	700 - 850
OPD3	GTCGCCGTCA	5	900 - 1950
OPD7	TTGGCACGGG	3	550 - 1900
OPD8	GTGTGCCCCA	5	850 - 2000
OPD18	GAGAGCCAAC	4	1200 - >3000
OPD20	ACCCGGTCAC	8	950 - 2900
OPN4	GACCGACCCA	7	600 - 2100
OPN16	AAGCGACCTG	4	350 - 2100
OPN18	GGTGAGGTCA	3	1200 - 1800
OPAF5	CCCGATCAGA	4	1050 - 1900
OPAF14	GGTGCGCACT	3	700 - 2800
Total		74	
ISSR primer			
SPS 1	(GAC)5	5	400 - 1031
SPS 2	(GTGC)4	9	550 - 1031
SPS 3	(GACA)4	7	400 - 1450
SPS 4	(AGG)6	6	325 - 1100
SPS 5	(GA)9T	6	800 - 1700
SPS 6	T(GA)9	2	475 - 850
SPS 7	(GTG)5	5	400 - 1200
SPS 8	(GGA)4	7	325 - 1400
Total		47	

Venkatachalam et al., 2007; Panda et al., 2007; Mohanty et al., 2008; Singh et al., 2010). ISSR markers are considered suitable to detect variations among tissue culture-produced plants (Leory et al., 2001; Rahman and Rajora, 2001; Joshi and Dhawan, 2007), since a simple sequence repeat-based primer targets the fast evolving hypervariable sequences (Tautz, 1989). 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). There are many factors like length of culture periods, genotype, and nature of explant, which could influence the stability of the tissue-cultured plants (Hammerschlag et al., 1987;

Nayak and Sen, 1998; Vendrame et al., 1999). But in our study, the length of culture period (more than two years) did not affect the genetic integrity of *Z. zerumbet.* Others (Salvi et al., 2002; Martins et al., 2004) have also reported results similar to this. Our study, in close agreement to Zuchi et al. (2002), reveals that the absence of DNA polymorphism in micropropagated *Z. zerumbet* could be due to the absence of DNA polymorphism in source mother plants.

The present study provides the first report on the genetic stability of micropropagated *Z. zerumbet* from sprouted buds of rhizomes using RAPD and ISSR analysis, which showed no remarkable changes in phenotypic characteristics and molecular profiling of all the regenerants analyzed in comparison with the source plants (control). Thus, we concluded that the protocol developed could be effectively used as a method for rapid micropropagation of genetically uniform clones of *Z.zerumbet* for commercial utilization, which will definitely satisfy its increasing market demand in future.

ACKNOWLEDGEMENT

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