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Micropropagation of *Zingiber rubens* and assessment of genetic stability through RAPD and ISSR markers

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Abstract

Protocol was developed for high frequency *in vitro* multiplication of an endemic species, *Zingiber rubens* Roxb. The sprouted buds of the rhizomes were cultured on Murashige and Skoog (MS) medium supplemented with 6-benzyladenine (BA; 0.5 - 5.0 mg dm^{-3}), indole-3-acetic acid (IAA; 0.5 -2.0 mg dm^{-3}), kinetin (KIN; 1.0 - 3.0 mg dm^{-3}), naphthaleneacetic acid (NAA; 0.5 - 1.0 mg dm^{-3}) and adenine sulphate (ADS; 80 - 100 mg dm^{-3}). MS basal medium supplemented with 3 mg dm^{-3} BA and 0.5 mg dm^{-3} IAA was optimum for shoot elongation. The elongated shoots (1 - 2 cm) were transferred to multiplication medium containing 2 mg dm^{-3} BA, 1 mg dm^{-3} IAA and 100 mg dm^{-3} ADS. The multiplication rate remained unchanged in subsequent subcultures. Upon *ex vitro* transfer, 85 % of plants survived. 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.

Additional key words: auxins, cytokinins, *ex vitro* transfer, genetic integrity, red ginger.

Introduction

Red ginger (*Zingiber rubens* Roxb.) is an endemic species of family *Zingiberaceae* (Jain and Prakash 1994) restricted to northeast India and to eastern Himalayas. The plant is used as an ornamental or for medicinal purposes. The plant propagates slowly producing maximum of 8 plants per rhizome in a year in the field. The endemic nature and slow natural propagation rate insists its conservation and propagation through tissue culture technique. *In vitro* technique can be used in propagation and conservation of endemic species either to produce new plants or as intermediate or long term storage system (Munoz 1995). Plant regeneration *in vitro* and reintroduction into natural habitat is one strategy for conservation of important plant species (Rout and Das 2002).

Periodic monitoring of the degree of genetic stability of *in vitro* conserved plants is of utmost importance for commercial utilization of true-to-type plants of the desired genotype. The assessment of the genetic integrity of *in vitro* grown regenerants in regular intervals can significantly reduce or eliminate the chance of occurrence of somaclonal variation (Larkins and Scowcroft 1981) at the early or late phase of culture. Of the various molecular markers random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) analysis are the simplest and quickest method for genetic stability assessment of *in vitro* grown plants. *In vitro* cultivation has been reported for many species of genus *Zingiber*, *i.e.*, *Z. officinale* (Bhagyalakshmi and Singh 1988, Balachandran *et al.* 1990, Kacker *et al.* 1993, Sharma and Singh 1997, Rout and Das 2002, Mohanty *et al.* 2008), *Z. cassumunar* (Poonasapaya and Kraisintu 1993), Z. *petiolatum* (Chang and Criley 1993), *Z. spectabile* (Faria and Illg 1995), *Z. wighitianum* *Z. montanum*, and *Z. zerumbet* (Tyagi *et al.*. 2006). In the present paper we report micropropagation, *in vitro* conservation and genetic stability of *in vitro* regenerated plantlets of ethnobotanically important *Zingiber rubens*.

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Abbreviations: ADS - adenine sulphate; BA - benzyladenine; IAA - indoleacetic acid; MS - Murashige and Skoog medium; NAA - napthaleneacetic acid; KIN - kinetin; RAPD - random amplified polymorphic DNA; ISSR - inter simple sequence repeats.
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Materials and methods

Healthy rhizomes of *Zingiber rubens* Roxb. collected from Kalimpong, West Bengal, India were planted in soil to initiate sprouting. The sprouted rhizomes were then removed from soil and washed properly in tap water. 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 11 - 12 min. After rinsing with sterile distilled water three times explants (10 - 12 mm) were inoculated to shoot induction Murashige and Skoog (1962; MS) medium containing 30 g dm^{-3} sucrose and different concentration of 6-benzyladenine (BA; 0.5 - 5.0 mg dm^{-3}), indole-3-acetic acid (IAA; 0.5 - 2.0 mg dm^{-3}) and kinetin (KIN; 1.0 - 3.0 mg dm^{-3}). The pH of the medium was adjusted to 5.7 before adding agar to it. It was then autoclaved at 121 ºC and 104 kPa for 20 min. The culture was maintained at temperature of 25 ± 1ºC, 16-h photoperiod (irradiance of 55 µmol m^{-2} s^{-1}. After six weeks, the shoots of length 25 - 30 mm were transferred to a multiplication medium containing in addition to BA, KIN and IAA also naphthaleneacetic acid (NAA; 0.5 - 1.0 mg dm^{-3}) and adenine sulphate (ADS; 80 - 100 mg dm^{-3}). *In vitro* multiplied shoots with well developed roots were separated and transferred to media containing 1 mg dm^{-3} BA. After four weeks, plantlets with shoots and roots were take out from the flasks and washed thoroughly. They were then transferred to pot containing soil and sand (1:1) and were kept in greenhouse for acclimatization for 2 - 3 weeks. For *in vitro* conservation, the plantlets were maintained in the same media up to two years with subculturing at an interval of 3 - 4 months. After acclimatization in a greenhouse the plants were grown in the field to maturity. All the experiments were repeated three times with a minimum of ten replicates.

Healthy and young leaves of *Z. rubens* 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 T_{10} E_1 buffer to 25 µg cm^{-3} for PCR amplification.

For RAPD analysis a total of 30 random primers were used out of which 18 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 94 ºC for 5 min for complete denaturation. The second step consisted of 42 cycles having three ranges of temperature, *i.e.*, 92 º for 1 min for denaturation of template DNA, 37 ºC for 1 min for primer annealing, 72 ºC for 2 min for primer extension, followed by running the samples at 72 ºC for 7 min for complete polymerization. For ISSR the same temperature profile was followed, but the primer annealing temperature was set at 5 ºC 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).

Data were subjected to analysis of variance for a factorial experiment. Critical differences (CD) were calculated to determine the statistical significance of different treatments.

Results and discussion

The explants remained dormant up to 15 d of inoculation, after which the initiation of shoot buds was observed from swelling bases of the explants. Further growth of shoot bud occurred when explants were again transferred to a fresh media (Fig. 1*B*,*C*). MS media containing BA (3 mg dm^{-3}) and IAA (0.5 mg dm^{-3}) were found to be optimum for shoot initiation where about 85 % of explants responded (Table 1). KIN showed poor response towards shoot initiation.

In vitro induced shoots were then subcultured to MS media containing various combinations of BA and KIN individually or in combination with IAA, NAA and ADS for shoot multiplication (Table 2). Of various combinations tried, MS medium with BA (2 mg dm^{-3}), IAA (1 mg dm^{-3}) and ADS (100 mg dm^{-3}) was optimum for shoot multiplication and root development (8.0 ± 0.13 shoots with 7.61 ± 0.18 roots per explant). Role of BA in shoot multiplication has been reported in other plant species (*e.g.* Dai *et al*. 2009, Goncalves *et al*. 2009, Malikarjuna and Rajendrudu 2009) including the species of *Zingiberaceae* (Ikeda and Tambe 1989, Balachandran *et al.* 1990, Smith and Hamil 1996, Rout *et al.* 2002,

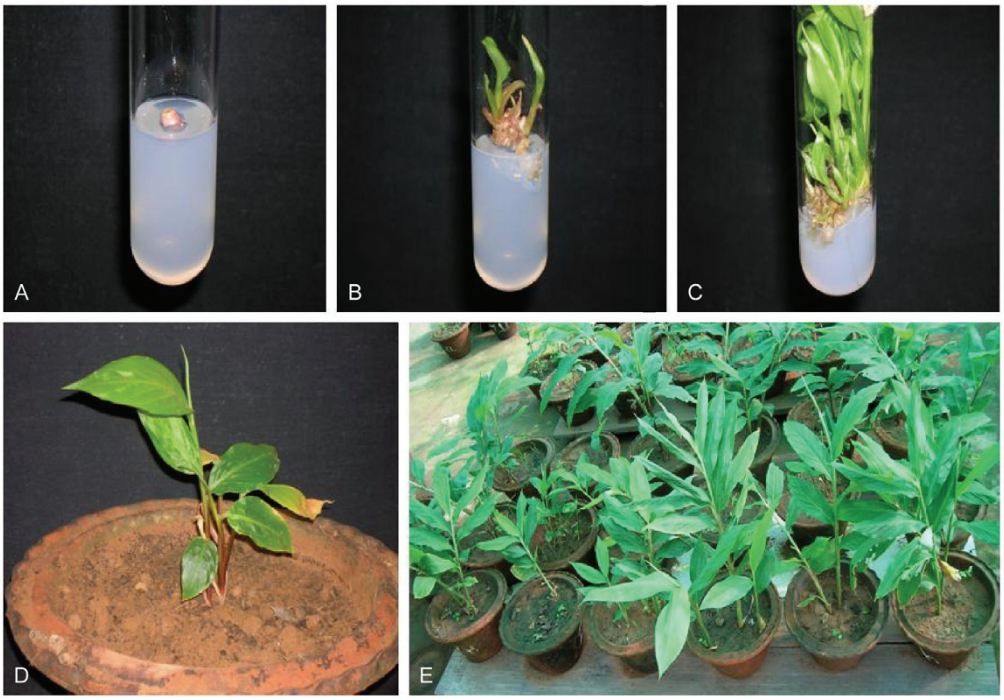

Fig. 1. *A* - Sprouted bud explant of *Zingiber rubens*. *B* - Induction of multiple shoots on MS medium with BA (3 mg dm^{-3}) and IAA (0.5 mg dm^{-3}) after 4 weeks. *C* - *In vitro* multiplication on MS medium with BA (2 mg dm^{-3}), IAA (1 mg dm^{-3}) and ADS (100 mg dm^{-3}) after 6 weeks. *D* - Potted plant in greenhouse 3 weeks after *ex vitro* transfer. *E* - Micropropagated plants growing in pots under natural condition for 12 weeks.

Table 1. Effect of BA, IAA and KIN [mg dm^{-3}] for *in vitro* shoot initiation of *Z. rubens*. Means ± SE, $n = 15$. Means having the same letter in a column were not significantly different at $P < 0.001$.

BA	KIN	IAA	Response [%]
0.5			19.06 ± 0.07a
1			26.40 ± 0.71a
3			64.46 ± 0.77b
5			46.00 ± 0.52b
-	1		32.67 ± 0.49a
-	3		33.20 ± 0.50a
0.5		0.5	43.93 ± 0.67a
1		0.5	72.60 ± 0.38c
3		0.5	84.80 ± 0.63c
5		0.5	47.26 ± 0.40b
-	1	0.5	56.40 ± 0.80b
-	3	0.5	35.93 ± 0.81a

Panda *et al.* 2007). Maximum number of root occurred in MS medium with 2 mg dm^{-3} KIN and 0.5 mg dm^{-3} IAA (Table 2). MS media containing KIN with NAA and IAA also showed response towards multiplication, *i.e.*, 5 to 6 shoots per explants, but the shoot were not healthy as after 15 - 20 d the leaves became curled with stunted growth.

The plantlets were then separated and maintained in MS media with BA (1 mg dm^{-3}). The plants could be conserved in this media for 30 months followed by subculturing at an interval of 120 d.

The fully developed plants with roots and shoots were transferred to pots containing soil and sand in the ratio 1:1 (Fig. 1*D*). Plants were kept in greenhouse and hardened for 2 - 3 weeks, after which they were transferred to field. About 85 % of plants were survived and grown to maturity (Fig 1*E*). Evaluation of morphological characteristics such as plant height, tiller number and rhizome yield of tissue culture regenerated *Z. rubens* showed no significant difference to that of conventionally grown mother plants.

Periodic assessment of genetic stability of the micropropagated plants was done through RAPD and ISSR analysis at 6 months interval up to 30 months. A total of 60 plantlets were analyzed taking minimum 12 plants from each culture period. 18 selected RAPD primers gave rise to a total of 109 scorable bands ranging from 350 to >3000 bp (Table 3).The number of bands for each primer varied from 3 - 9 with an average of 6 bands per primer. A total of 6540 bands (total number of bands × number of explants analyzed) were generated giving all of which were monomorphic (Fig. 2*A*). The highest number of bands obtained was 9 in case of primers OPA18, OPD20 and OPAF5 and the lowest number of bands, *i.e.* 3, was obtained in case of primers OPD3, OPD7 and OPAF14. In ISSR analysis, 8 selected primers produced a total of 42 scorable bands ranging from

Table 2. Effect of different growth regulators on shoot and root multiplication in *Z. rubens*. Means ± SE, $n = 15$. Means having the same letter in a column were not significantly different at $P < 0.001$.

Growth regulators [mg dm^{-3}]	Shoot number [explant^{-1}]	Shoot length [mm]	Root number [explant^{-1}]	Root length [mm]
BA (1.0) + IAA (0.5)	2.87 ± 0.19a	5.47 ± 0.07b	7.87 ± 0.21b	2.47 ± 0.07a
BA (1.0) + IAA (0.5) + ADS (100)	5.73 ± 0.11b	5.11 ± 0.02b	8.40 ± 0.16b	4.04 ± 0.09a
BA (2.0) + IAA (1.0) + ADS (100)	8.00 ± 0.13c	6.52 ± 0.04c	7.93 ± 0.26b	8.27 ± 0.05c
BA (1.0) + KIN (0.5)	5.13 ± 0.16b	4.87 ± 0.06b	8.53 ± 0.13b	6.87 ± 0.03b
BA (1.0) + NAA (0.5) + ADS (100)	2.53 ± 0.16a	4.22 ± 0.02a	3.46 ± 0.13a	5.24 ± 0.05b
BA (2.0) + NAA (1.0) + ADS (100)	3.06 ± 0.11a	3.93 ± 0.01a	3.26 ± 0.15a	4.80 ± 0.02a
KIN (0.5) + IAA (0.5)	2.60 ± 0.13a	3.36 ± 0.03a	2.47 ± 0.13a	2.26 ± 0.07a
KIN (1.0) + IAA (0.5)	5.73 ± 0.18b	5.09 ± 0.02b	5.40 ± 0.21a	5.20 ± 0.05b
KIN (2.0) + IAA (0.5)	3.53 ± 0.13a	5.86 ± 0.03c	9.20 ± 0.14c	5.80 ± 0.07b
KIN (0.5) + NAA (0.5)	5.73 ± 0.22b	5.38 ± 0.09b	5.61 ± 0.21a	7.90 ± 0.05c
KIN (1.0) + NAA (0.5)	3.33 ± 0.12a	4.29 ± 0.6a	4.53 ± 0.16a	7.19 ± 0.05b

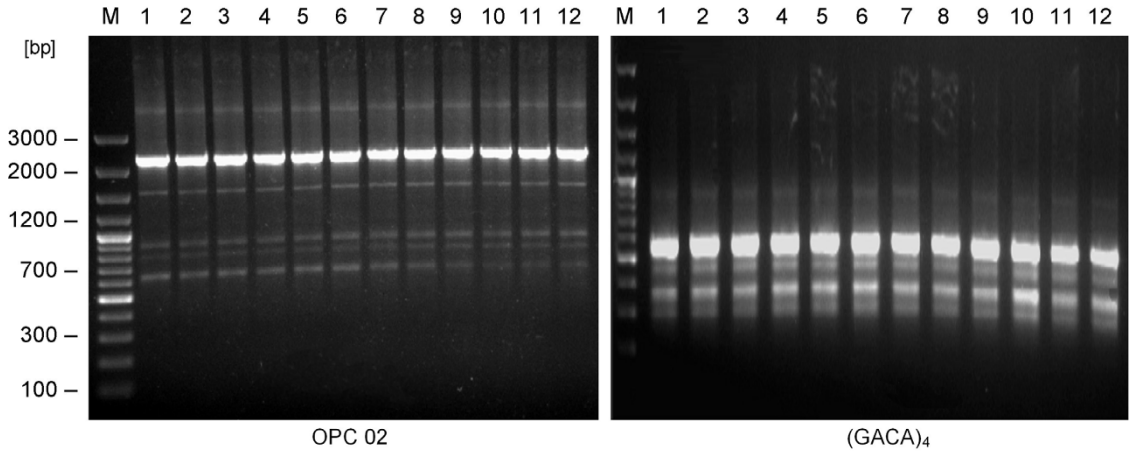

Fig. 2. RAPD (OPC02) and ISSR $(GACA)_4$ banding pattern in micropropagated plants and field grown mother plant of *Z. rubens* determined after 24-month storage (*lane 1* - mother plant, *lanes 2 to 12* - micropropagated plants, M - ladder).

Table 3. RAPD banding pattern of both micropropagated and field grown mother plants after 2 years.

Primers	Sequence	Amplicons [bp]	Total bands
OPA04	AATCGGGCTG	1900-575	5
OPA07	GAAACGGGTG	2100-350	6
OPA09	GGGTAACGCC	2100-575	4
OPA18	AGGTGACCGT	2300-600	9
OPC02	GTGAGGCGTC	3000-650	6
OPC05	GATGACCGCC	2300-950	8
OPC11	AAAGCTGCGG	1300-350	6
OPD03	GTCGCCGTCA	2000-450	3
OPD07	TTGGCACGGG	1400-600	3
OPD08	GTGTGCCCCA	2000-450	8
OPD18	GAGAGCCAAC	2000-450	8
OPD20	ACCCGGTCAC	2300-350	9
OPN04	GACCGACCCA	1800-550	7
OPN16	AAGCGACCTG	1450-475	7
OPN18	GGTGAGGTCA	1050-550	4
OPAF05	CCCGATCAGA	2000-350	9
OPAF14	GGTGCGCACT	1450-450	3
OPAF15	CACGAACCTC	1100-400	4

250 to 1900 bp (Table 4). For each primer, the number of amplified bands varies from 2 - 8 and a total of 2520 bands were generated all of which were monomorphic in nature (Fig. 2*B*). The highest number of bands, *i.e.* 8, was generated by the primer SPS 08, whereas the lowest number of bands, *i.e.* 2, was recorded for the primer SPS 02.

Table 4. ISSR banding pattern of micropropagated and field grown mother plants in *Z. rubens* after 2 years.

Primers	Sequence	Amplicons [bp]	Total bands
SPS 01	$(GAC)_5$	700-250	6
SPS 02	$(GTGC)_4$	700-300	2
SPS 03	$(GACA)_4$	1900-400	5
SPS 04	$(AGG)_6$	1100-550	6
SPS 05	$(GA)_9T$	1100-450	6
SPS 06	$T(GA)_9$	850-475	5
SPS 07	$(GTG)_5$	600-400	4
SPS 08	$(GGA)_4$	1100-325	8

The present study provides the first report on the genetic stability of micropropagated *Z. rubens* from sprouted buds of rhizomes using RAPD and ISSR analysis. Genetic integrity by RAPD and ISSR analysis has been reported earlier in many plant species (Rout *et al.* 2002, Joshi and Dhawan 2007, Panda *et al.* 2007, Venkatachalam *et al.* 2007, Mohanty *et al.* 2008). 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. rubens*. Results similar to this have also been reported by others (Salvi *et al.* 2002, Martins *et al.* 2004). Our study, in close agreement to Zuchi *et al.* (2002), reveals that the absence of DNA polymorphism in micropropagated *Z. rubens* could be due to the absence of DNA polymorphism in source mother plants.

In the present study we have developed a protocol for the micropropagation of *Z. rubens*. RAPD and ISSR markers show the genetic stability of propagated plants. We conclude that the protocol developed could be used as a method for large scale propagation and conservation.

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