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EVALUATION OF GENETIC FIDELITY OF *IN VITRO* PROPAGATED GREATER GALANGAL (*ALPINIA GALANGA* L.) USING DNA BASED MARKERS

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ABSTRACT : Tissue cultured *Alpinia galanga* plantlets were subjected to assessment of genetic stability considering the fact that associated *in vitro* stress might result in breakdown of control mechanism causing instability of the genome. We have used two DNA based molecular markers to assess the genetic fidelity of *in vitro* regenerated *Alpinia galanga* L. through axillary buds from unsprouted rhizomes. The sprouted buds upon transfer to medium containing Kinetin (3 mg/l), Benzyladenine (3 mg/l) and Napthalene aceticacid (1.0 mg/l) exhibited maximum number of shoot multiplication as 15.6±0.2. Out of 30 RAPD and 13 ISSR primers screened, only 12 random amplified polymorphic DNA (RAPD) and 9 inter simple sequence repeats (ISSR) primers produced clear reproducible and scorable bands. All banding profiles from micropropagated plants were monomorphic and similar to the mother plant. A similarity matrix based on Jaccard's coefficient revealed the pair-wise value as 1 between the mother and *in vitro* regenerants. After 2 years of culture *in vitro*, plantlets were transplanted to the field and evaluation of phenotypic characteristics was done. This study is of high significance as these could be commercially utilized for large scale production of true-to-type plantlets in *Alpinia galanga.*

Key words: *Alpinia galanga*, *in vitro* propagation, genetic fidelity, DNA markers

INTRODUCTION

Alpinia galanga Linn. is a perennial aromatic rhizomatous herb and an important crop plant of family Zingiberaceae which is cultivated in India, China, Thailand, Malaysia and Indonesia (Saritnum and Sruamsiri, 2003; Jirovetz *et al.,* 2003; Nan *et al.,* 2004; Jatoi *et al.,* 2006). Many *Alpinia* species are appreciated for their medicinal properties and are also used in traditional medicines as a spasmolytic, hypotensive, anti-emetic, anti-oxidant, anti-inflammatory, bacteriastatic, fungistatic effects in India, China and other regions (Nan *et al.,* 2004; Zaeoung *et al.,* 2005; Tachakittirungrod and Chowwanapoonpohn, 2007; Pompimon *et al.,* 2009; Mahae and Chaiseri, 2009). The rhizomes of *A. galanga* is an essential spice used for flavouring food, smells as cardamom so mainly used as spices and condiment along with its medicinal properties. The rhizome is used against rheumatid arthritis, bronchial catarrh, ulcers, whooping colds in children, throat infections, intermittent fevers, incontinence control, vomiting, stomachache and indigestion. Due to the presence of essential oil, the rhizomes are used in bronchial troubles and as a carminative. Phytochemical constituents of rhizome have antitumour, antiulcer, anticalculi activity (Borthakur *et al.,* 1999). The rhizomes of *A. galanga* are also reported to have anti-HIV agents (Ye and Li, 2006). Most of the medicinal plants are collected from wild areas and their commercial exploitation has resulted in receding the population in their natural habitat (Balakrishnan *et al.,* 2009). Consequently, cultivation of these plants is urgently needed to ensure their availability to the industry as well as to people associated with traditional system of medicine. *In vitro* conservation of micropropagated plants is a safe method to protect the species from risk of natural vagaries (Borthakur *et al.,* 1999). If steps are not taken for their conservation, cultivation and mass propagation, they may be lost from the natural vegetation for ever. Many Zingiberaceae species are reported for its *in vitro* multiplication which is an easy and safe method for production of true-to-type plants within a short span of time (Selvakkumar *et al.,* 2007).

True-to-type clonal fidelity is one of the most important pre-requisites in the micropropagation of plant species. The occurrence of cryptic genetic defects arising via somaclonal variation in the regenerates can seriously limit the broader utility of the micropropagation system (Salvi *et al.,* 2001). It is, therefore, imperative to establish genetic uniformity of micropropagated plants to confirm the quality of the plantlets for its commercial utility. Few reports (Borthakur *et al.,* 1999; Saritnum and Sruamsiri, 2003) available so far on *in vitro* multiplication of *A.galanga* exclude any work on genetic stability analysis of regenerants. Of the various DNA based molecular markers random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) markers are very simple, fast, cost-effective, highly discriminative and reliable. They require only a small quantity of DNA sample and they do not need any prior sequence information to design the primer. Thus, they are suitable for the assessment of the genetic fidelity of *in vitro* raised clones. In this study, we assessed the genetic stability of *in vitro* raised *A.galanga* plants using RAPD and ISSR markers. This technique would facilitate an alternative method for large scale production and successful establishment of genetically stable plants before it is released for commercial purpose.

MATERIALS AND METHODS

Plant material and cultural conditions

The rhizomes were collected from the wild areas of Phulbani, Orissa and was grown in the medicinal plant garden of Center Of Biotechnology, Siksha O Anusandhan University, Bhubaneswar. The axillary bud explants collected from disease-free plants were thoroughly washed in running tap water with a neutral liquid detergent (Extran, Merck) for 3 to 5 minutes. These explants were cut into convenient sizes after removal of the leaf sheaths. The cut pieces were surface sterilized with 0.1% mercuric chloride for 5 to 7 minutes in a laminar flow cabinet and rinsed 3 to 4 times in sterile distilled water to remove the traces of sterilants prior to inoculation. Surface disinfected explants were inoculated on basal Murashige and Skoog (MS) medium supplemented with combination of KIN (3 mg/l), BA (1-3 mg/l), IAA (0.5-1.0 mg/l), IBA (1.0 mg/l) NAA (0.5-2.0 mg/l) and Ads (100 mg/l) for culture establishment. The sucrose amount in the media was 30 gm/lt and agar 0.8% was used as basal. All experiments were conducted in ten replicas for each treatment. The pH of the medium was adjusted to 5.7 before adding agar and was autoclaved at 121^0 C and 105 kg/cm^2 of pressure for 20 minutes. All the cultures were incubated at 25 ± 1^0C under white fluorescent light with 50µ mole m^{-2} s^{-2} light intensity during a photoperiod of 16:8 h light and dark cycles. The *in vitro* derived shoots were cultured on medium [MS +KIN (3 mg/l) + BA (3 mg/l) + NAA (1 mg/l)] for proliferation and multiplication. Further these plantlets were cultured on rooting media containing Benzyladenine (1 mg/l) and Indolebutyric acid (1 mg/l) and the maximum number of root produced was 7.8 ± 0.3.

Field transfer of regenerants and analysis of morphological characteristics

The plantlets were hardened in earthen pots containing soil, cowdung and sand mixture in 1:1:1 ratio, then kept in greenhouse for acclimatization for 15 days and then to field for attending maturity. The percentage of field survival was noted. Micropropagated plants (TC) and conventionally propagated plants (CP) were compared for various phenotypic and molecular characters. After 6 months of transplantation, the morphological characters such as plant height, tiller number, leaf biomass, leaf number, leaf length, leaf breadth and rhizome yield was recorded. The mean value (Mean) as well as its standard error (SE) were calculated for each characters.

DNA extraction and PCR amplification conditions

The genomic DNA of *A.galanga* was isolated by following the protocol of Doyle and Doyle (1990) from both *in vitro* and *ex vitro* grown mother plants.

Clonal fidelity of *in vitro* raised regenerants was tested using RAPD and ISSR markers. For this purpose, 10 *in vitro* raised hardened plants were chosen randomly from the population and compared with the mother plant from which the explants were taken. Thirty RAPD (OperonTech, Almeda, USA) and thirteen ISSR (Bangalore Genei Pvt. Ltd, Bangalore, India) primers were used for initial screening. The RAPD and ISSR analysis was performed as per the methodology given by Williams *et al.* (1990) and Zietkiewicz *et al*., (1994). PCR amplifications were carried out in a total volume of 25µl containing 25ng of genomic DNA as template,2.5ml of 10x assay buffer(100 mM Tris Hcl, pH 8.3, 500mM Kcl and 0.1% gelatin), 1.5 mM $Mgcl_2$, 200µM dNTPs (Bangalore Genei, India), 0.5 unit (U) of Taq polymerase (Bangalore Genei, India) and 15ng of primer (Bangalore Genei, India). The primers showing polymorphic band were used for analysing the clonal fidelity of micropropagated plants. PCR amplification was performed in a DNA thermal cycler (Gene Amp PCR system 9700, Applied Biosystems, CA, USA), which was programmed for initial DNA denaturation at 94^0C for 5 min, followed by 42 cycles of 1 min denaturation at 92^0C, 1 min annealing (temperature specific to the primer) at 37^0C and 2 min extension at 72^0C, with a final extension at 72^0C for 7 min. Amplified products were resolved by electrophoresis on 1.5% and 2% agarose gel for RAPD and ISSR in TAE buffer (40mM Tris base, 20mMsodium acetate, glacial acetic acid to pH 7.2) stained with ethidium bromide (0.5µg μl^{-1}) for 3h at 60 volts and photographs were taken by using the Gel Documenting system (Bio-Rad, USA).

Data scoring and 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. Consistent, well-resolved fragments in the size range of 100 bp to 3000bp were manually scored. Each band was treated as a marker. The scoring of bands was done on the basis of their presence ('1') or absence ('0') in the gel and missing data was denoted by '9'. The genetic associations were evaluated by calculating the Jaccard's similarity coefficient for pair-wise comparisons based on the proportion of shared bands produced by the primers. The similarity matrix was subjected to the cluster analysis of unweighted pair group method with arithmetic averages (UPGMA) and a dendrogram was generated by using NTSYS-pc version 2.1 software (Rohlf, 2000).

RESULTS AND DISCUSSION

In vitro shoot multiplication and field transfer of regenerants

The axillary buds from unsprouted rhizomes of conventionally grown plants were used as explant (Fig.1a) and inoculated to MS media containing varying combinations of KIN (3 mg/l), BA (1-3 mg/l), IAA (0.5-1.0 mg/l), IBA (1.0 mg/l) NAA (0.5-2.0 mg/l) and Ads (100 mg/l). Effect of different plant growth regulators on sprouting of these axillary buds was observed. Of the various media tried, i*n vitro* sprouting was relatively more on media containing BA (3 mg/l) alone but without any multiplication in the same. The multiplication of shoot buds was seen on the media containing KIN, BA and NAA combinations. Proliferations of shoots were observed in different culture conditions as mentioned in Table-1. Among the various concentrations maximum multiplication of shoots were found in MS supplemented with KIN (3 mg/l), BA (3 mg/l) in combination with NAA (1.0 mg/l) (Table 1). On this media, the optimum number of shoots formed was 15.6 ± 0.2, shoot length was 10.4 ± 0.3 and root number was 4.3 ± 0.5. Similar protocol was established by Kochuthressia *et al.* (2010), in which multiple shoots upto a mean of 6.42 shoots per explant in *Alpinia purpurata* using rhizome bud explants was found on MS medium supplemented with BA (3.0 mg/l) and KIN (2.0 mg/l).

According to Borthakur *et al*. (1999) KIN (3 mg/l) alone induced a mean of 8 shoots as well as roots within 8 weeks. In our study the best shoot multiplication was observed at KIN (3 mg/l), BA (3 mg/l) and NAA (1 mg/l) with a maximum number of 15 shoots within 8 weeks, media containing BA (1 mg/l) and IBA (1 mg/l) produced maximum number of roots as 7.8 ± 0.3 (Fig.1b). Selvakkumar *et al*. (2007) reported a protocol for *in vitro* micropropagation of *Alpinia officinarum* through rhizome bud explants. The optimum shoot multiplication in their study was observed in MS containing KIN (3 mg/l) and NAA (1 mg/l) which gave rise to 11 shoots per explants and rooting was more suitable in half strength MS and IBA (0.5 mg/l) producing an average of 7 roots. The effectiveness of IBA in rooting has been reported in many other medicinal plants (Sreekumar *et al*., 2000; Abrie and Vanstaden, 2001) and the use of half strength MS medium for rooting by Pence and Soukup (1993). The cultures of *A.galanga* were maintained in same media with regular sub culturing at 2 months interval. Multiplication rate remain unchanged even after 2 years of culture. During the planting season same plantlets were transferred to the pots and had luxuriant growth.

Table 1: *In vitro* shoot multiplication in *Alpinia galanga* using different plant growth regulators

Serial No.	MS Media + Growth regulators (mg/l)	% of shoot initiation (Mean± SE)*	No.of shoots/explant (Mean ± SE)*	Shoot length (Mean± SE)*	No.of roots/explant (Mean ± SE)*
1	BA(3)	87.8 ± 0.4 ^{d}	5.4 ± 0.2 ^{b}	4.0 ± 0.2^{b}	1.6 ± 0.2 ^{a}
2	KIN(3)	70.2 ± 0.8 ^{c}	6.4 ± 0.2 ^{c}	4.3 ± 0.5^{b}	4.6 ± 0.5 ^{b}
3	BA(1)+IAA(0.5)	29.8 ± 0.8 ^{a}	2.2 ± 0.2 ^{a}	2.8 ± 0.1^{a}	2.2 ± 0.2 ^{a}
4	BA(1)+IAA(1)	38.4 ± 0.8 ^{a}	2.4 ± 0.2 ^{a}	7.3 ± 0.5^{c}	3.4 ± 0.2 ^{b}
5	BA(3)+IAA(0.5)	78.1 ± 0.4 ^{c}	4.4 ± 0.2 ^{b}	11.1 ±0.3^{d}	3.0 ± 0.4 ^{b}
6	BA(3)+IAA(1)	80.5 ± 0.8 ^{d}	3.4 ± 0.2 ^{a}	8.0 ± 0.3^{c}	5.6 ± 0.4 ^{c}
7	BA(1)+NAA(0.5)	31.1 ± 0.5 ^{a}	3.2 ± 0.2 ^{a}	10.6 ± 0.3^{d}	4.0 ± 0.3 ^{b}
8	BA(3) +NAA(0.5)	75.0 ± 0.8 ^{c}	8.0 ± 0.3 ^{d}	8.1 ± 0.1 ^{c}	2.0 ± 0.0 ^{a}
9	BA(1)+IBA(1)	20.6 ± 0.8 ^{a}	2.2 ± 0.2 ^{a}	4.8 ± 0.4^{b}	7.8 ± 0.3 ^{d}
10	KIN(3)+NAA(0.5)	51.5 ± 0.3 ^{b}	7.2 ± 0.3 ^{c}	11.0 ± 0.7^{d}	6.0 ± 0.3 ^{c}
11	KIN(3)+NAA(1)	54.6 ± 0.8 ^{b}	8.2 ± 0.3 ^{d}	11.4 ± 0.6^{d}	4.2 ± 0.3 ^{b}
12	KIN(3)+BA(3)+NAA(0.5)	62.7 ± 0.6 ^{c}	12.4 ± 0.2 ^{f}	12.8 ± 0.3^{e}	5.0 ± 0.3 ^{c}
13	KIN(3)+BA(3)+NAA(1)	67.1 ± 0.6 ^{c}	15.6 ± 0.2 ^{g}	10.4 ± 0.3^{d}	4.3 ± 0.5 ^{b}
14	KIN(3)+BA(3)+NAA(2)	50.5 ± 0.2 ^{b}	6.9 ± 0.6 ^{c}	9.2 ± 0.1^{d}	3.2 ± 0.6 ^{b}
15	BA(3)+IAA(1)+ADS(100)	47.0 ± 0.6 ^{b}	3.2 ± 0.3 ^{a}	4.0 ± 0.2^{b}	2.6 ± 0.4 ^{a}

* a-g: Mean having the same letter in a column were not significantly different at $p < 0.05$ level. Data represent the mean of 15 replicates for each treatment.

The full grown plants with shoots and roots were transferred to pots containing soil, cowdung and sand mixture in 1:1:1 ratio (Fig.1d). Plants were hardened for seven days and were then transferred to green house and subsequently grown in normal field condition. These plants were planted and subjected to RAPD and ISSR analysis for assessing genetic stability. About 85% of plants survived and grown to maturity in field condition.

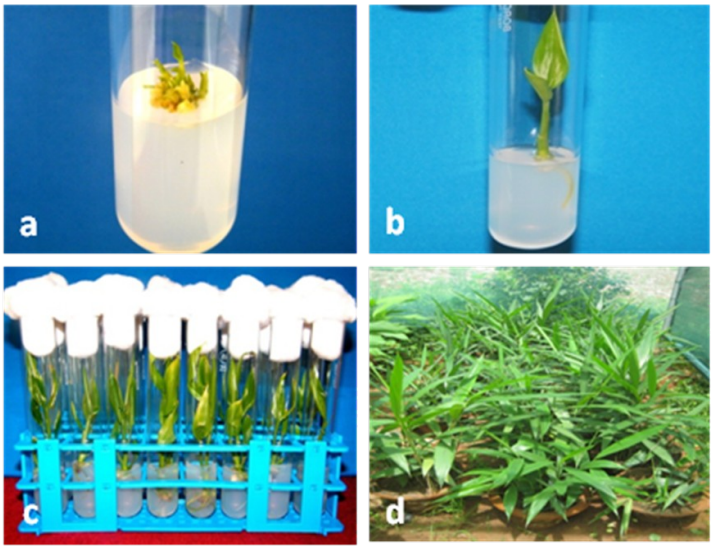

Fig.1. (a) Explant showing shoot initiation from axillary bud explant in *A.galanga* (b) Regenerated plants of *A.galanga* with shoots and roots (c) Multiplication of plantlets *in vitro* condition (d) Micropropagated plants of *A.galanga* growing under field conditions

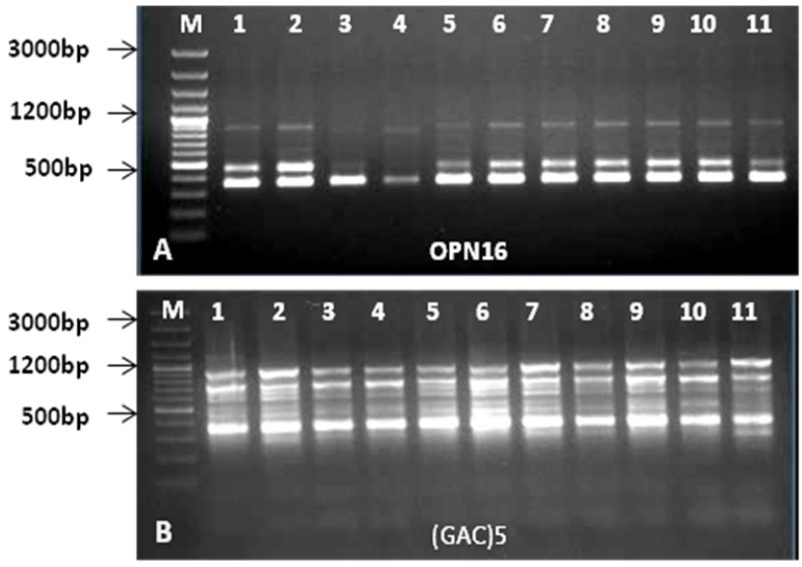

Fig 2. A & B: RAPD and ISSR banding pattern in both micropropagated and field grown mother plants of *Alpinia galanga* (Lane 1, mother plant; Lane 2-11 micropropagated plants and M-marker).

Table 2(A): List of primers, their sequences, total number of bands and size of amplified fragments generated by RAPD primers in both micropropagated and field grown mother plants of *Alpinia galanga*

Primer	Sequence	Total number of bands	Range of amplicons [bp]
OPA4	ATCGGGCTG	4	500 – 2000
OPA8	TGACGTAGG	6	800 – 2900
OPA9	GGGTAACGCC	4	800 – 2000
OPA18	GGTGACCGT	2	500 – 1500
OPC2	TGAGGCGTC	5	700 – 1900
OPC5	ATGACCGCC	2	1200 – 2000
OPD8	GTGTGCCCCA	2	700 – 2000
PD18	AGAGCCAAC	5	600 – 2800
PD20	ACCCGGTCAC	6	500 – 2000
OPN4	GACCGACCCA	5	600 – 1200
OPN16	AAGCGACCTG	3	375 – 900
OPN18	GGTGAGGTCA	4	300 – 900
Total		**48**	

Table 2(B): List of primers, their sequences, total number of bands and size of amplified fragments generated by ISSR primers in both micropropagated and field grown mother plants of *Alpinia galanga*

Primer	Sequence	Total number of bands amplified	Range of amplicons [bp]
SPS1	(GAC)5	6	300 – 900
SPS 2	GTGC)4	9	300 – 2000
SPS 3	GACA)4	9	325 – 1500
SPS 4	(AGG)6	11	275 – 1031
SPS 5	(GA)9T	3	300 – 1031
SPS 6	T(GA)9	8	400 – 1100
SPS 7	(GTG)5	11	300 – 900
SPS 8	(GGA)4	8	300 – 1500
SPS 9	(CAA)5	5	800 – 1500
	Total		**70**

Morphological analysis of ex vitro grown somaclones

A problem often associated with *in vitro* regenerated plants is the occurrence of high rates of somatic mutation (Rani *et al*., 1995; Wang *et al.,* 2000). Thus, to assess the genetic stability, we have evaluated both tissue culture derived and conventionally grown *A.galanga* through morphological and molecular analysis. For this purpose, both tissue culture-derived and conventionally cultivated mother plants were grown in the field under the same environmental conditions. Tissue culture-derived plants were assessed for their stability by comparing various phenotypic characters with those of conventionally grown *A.galanga*.

The result of the present study reveals difference in mean value of any of the characters as plant height, tiller number, leaf biomass, leaf number, leaf length, leaf breadth and rhizome yield studied between the two groups of plant (Table 3). It was found that higher value in mean and less standard error was observed in culture-derived plants than in conventionally grown plants, indicating that culture-regenerated plants were more uniform. 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 compared to the field-cultivated clones. Hatano *et al.* (1988) also observed a high uniformity in the alkaloid content of culture-derived somaclones of *Aconitum carmicheli* compared to the field-cultivated clones.

Table 3: Comparison of different phenotypic characteristics between conventionally propagated plants and culture regenerated plants of *Alpinia galanga*

Characters	**Conventionally propagated plants (Mean± SE)***	**Micropropagated plants (Mean± SE)***
Plant height (cm)	73 ± 2.54	66 ± 1.2
Tiller number/plant	15.6 ± 1.18	21.0 ± 0.9
Leaf biomass(gm)/plant	28.4 ± 2.50	37.6 ± 1.2
Leaf number	55.8 ± 2.0	75.6 ± 1.0
Leaf length	34.6 ± 2.03	36.2 ± 0.8
Leaf breadth	4.8 ± 0.6	5.2 ± 0.6
Leaf oil (%)	0.23 ± 0.02	0.35 ± 0.01
Rhizome yield (gm)/plant	84.2 ± 1.9	98.3 ± 1.1
Rhizome oil (%)	0.38 ± 0.03	0.46 ± 0.01

Assessment of genetic stability

RAPD and ISSR analysis was done up to30 months in an interval of 6 months. A total of 30 RAPD primers were used for initial screening with the mother plant of *A.galanga* but only 12 RAPD primers gave clear and reproducible bands. The number of scorable bands for each RAPD primer varied from 2 [(OPA18, OPC05 and OPD08)] to 6 [(OPA08 and OPD20)] (Table-2). The 12 RAPD primers produced 48 distinct and scorable bands, with an average of 4 bands per primer. Each primer generated a unique set of amplification products ranging in size from 300bp (OPN18) to 2900bp (A8). No polymorphism was detected during the RAPD analysis of *in vitro* raised clones (Fig.2). Shoot multiplication through axillary bud proliferation maintains genetic stability in tissue culture generated plants rather than the plants regenerated through adventitious bud explant (Balachandran *et al*., 1990; Shenoy and Vasil, 1992; Joshi and Dhawan, 2007). Genetic assessment of micropropagated plants by RAPD markers has been reported by many workers (Rani *et al.,* 1995; Rout and Das, 2002; Martins *et al*., 2004; Venkatachalam *et al*., 2007). Geetha (2002) studied on RAPD profiling of *in vitro* conserved ginger using 10 operon random primers which detect no polymorphism between the conserved lines in any of the primers tested, indicating the genetic stability. Bhowmik *et al.* (2009) reported analysis of genetic uniformity using RAPD markers in micropropagated *Mantisia spathulata*.

ISSR markers are considered suitable to detect variations among micropropagated plants since a simple sequence repeat targets the fast evolving hypervariable sequences (Tautz 1989; Leory *et al.,* 2001; Rahman and Rajora, 2001; Joshi and Dhawan, 2007). Out of 13 ISSR primers used in the initial screening, 9 of them produced clear and reproducible bands. The optimum annealing temperature for ISSR markers varied from 35 to 55^0C (Table-3). The nine ISSR primers produced 70 distinct and scorable bands in the size range of 300bp [$(GAC)_5$, $(GTGC)_4$, $(GA)_9T$, $(GTG)_5$ and $(GGA)_4$] to 2000bp $(GTGC)_4$ (Table-3). The number of scorable bands for each primer varied from 3 $(GA)_9T$ to 11 [(AGG)6 and $(GTG)_5$] with an average of 7.7 bands per primer. All banding profiles from micropropagated plants were monomorphic and similar to those of the mother plant (Fig.3).

Two PCR based techniques, RAPD and ISSR, were used to test clonal fidelity because of their simplicity and cost effectiveness. The use of two markers, which amplify different regions of the genome, allow better chances for the identification of genetic variations in the clones (Martin *et al*., 2004). The ISSR primers are now proved to be much more efficient in assessing the genetic integrity among clonally propagated plants as reported by many workers in different species (Zietekiewicz *et al.,* 1994; Bhatia *et al.,* 2009; Mohanty *et al.,* 2010; Bhatia *et al.,* 2011). Martins *et al.* (2004) reported genetic homogeneity of almond plantlets regenerated through axillary branching after 4 years and six years of *in vitro* multiplication. A homogeneity in amplification profile was reported for all micropropagated plantlets in *Swertia chirayita* through ISSR marker assay by Joshi and Dhawan, (2007). The number of bands in the study generated per primer was greater in ISSR (7.7) than RAPD (4.0). These differences could possibly be due to the high melting temperature for the ISSR primers, which permits much more stringent annealing conditions and consequently more specific and reproducible amplification. A total of 2400 and 3500 bands (number of plants analysed x number of scorable bands in RAPD and ISSR primers) were generated from the mother plant and *in vitro* raised clones. All banding profiles from micropropagated plants were monomorphic and similar to those of the mother plant (Fig. 2A, 2B). A similarity matrix based on Jaccard's coefficient revealed that the pair-wise value between the mother plant and the plantlets derived from different explants was 1, indicating 100% similarity. An UPGMA dendrogram was generated from the Jaccard's similarity values using NTSYS-pc software. A phenetic dendrogram based on UPGMA analysis further confirmed the true-to-type nature of clones. Similar results were obtained by Sreedhar *et al.* (2007) during the clonal fidelity analysis of long-term micropropagated shoot cultures of vanilla (*Vanilla planifolia* Andrews) by using RAPD and ISSR markers.

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 micropropagation. 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).

The present study provides the first report on the genetic fidelity of micropropagated *Alpinia galanga* obtained from axillary bud explants using RAPD and ISSR analysis. No variability was detected among the tissue cultured plantlets, hence we concluded that the protocol developed could be effectively used for rapid micropropagation and commercial utilization of *Alpinia galanga* without much risk of genetic instability.

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