African Journal of Biotechnology Vol. 8 (13), pp. 2933-2938, 6 July, 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

Genetic fidelity in micropropagated plantlets of *Ochreinauclea missionis* an endemic, threatened and medicinal tree using ISSR markers

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Accepted 5 January, 2009

Inter simple sequence repeat (ISSR) markers were employed to determine the genetic fidelity of *Ochreinauclea missionis* plantlets multiplied *in vitro* by using nodal segments. Thirty two ISSR primers were screened, of which twenty nine primers generated total of 183 clear, distinct and reproducible bands. Among 183 bands, 178 bands were monomorphic (97.2%) and 5 bands were polymorphic patterns (2.73%). Although minor morphological variations recorded in the micropropagated plants, the developed ISSR profiles were typical to that of mother plant. A UPGMA dendrogram was constructed to show the genetic similarity among the 22 plants (21 micropropagated plants and 1 donor mother plant). In the present study, molecular profiling by using ISSR markers proved to be a reliable method for assessing genetic stability of micropropagated plants. The developed *in vitro* plants which ascertained stability can be reintroduced back to its original habitat for conservation purpose.

Key words: Medicinal tree, *Ochreinauclea missionis,* genetic fidelity, ISSR analysis.

INTRODUCTION

Ochreinauclea missionis (Wall. ex G. Don) Ridsd. locally known as 'Jalamdasa', belongs to the family Rubiaceae. It is a medium sized evergreen tree endemic to Central and Southern Western Ghats of peninsular India. These trees are found growing along the river bank side of Seetha Nadhi in Udupi District, Karnataka, India. It is very much exploited by the local people for its purported medicinal value. The powdered bark and its decoction are used for curing cutaneous diseases like leprosy, ulcers and as an effective purgative (Kirtikar and Basu, 1975). Root and root bark are employed in treating rheumatism, paralysis, skin diseases, dropsy, eye diseases, constipation, piles, jaundice, fever, edema, hepatic and haemophilic disorders (Nayar and Sastry, 1990). *O. missionis* plants are reported as rare due to dwindling of natural forest as a result of construction of dams, hydroelectric projects, roads and agricultural purposes. To our knowledge, there have been no reports on the genomic stability or variation of *O. missionis* regenerated plants comparing to its mother plant however, the vegetative propagation and *in vitro* regeneration through nodal explants has been reported (Jose et al., 1998; Dalal and Rai, 2001).

Plant tissue culture is recognized as one of the key areas of biotechnology because of its potential use to regenerate elite and conserve valuable plant genetic resources and scaling up of any micropropagation protocol is severely hindered due to incidences of somaclonal variations (Larkin and Scowcroft, 1981). Several techniques have been developed to assess the genetic purity of tissue culture raised plants such as morphological descriptions, physiological supervisions, cytological studies, isozymes (Gupta and Varshney, 1999), field assessment and molecular studies (Devarumath et al., 2007). Somaclonal variation mostly occurs as response to the stress imposed on the plant in culture conditions and is manifested in the form of DNA methylations, chromosome rearrangements, and point mutations (Phillips et al., 1994). Molecular techniques are at present powerful and valuable tools used in analysis of genetic fidelity of *in*

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Abbreviations: CTAB, Cetyl trimethylammonium bromide; **PCR,** polymerase chain reaction; **ISSR,** inter-simple sequence repeat; **UPGMA,** unweighted pair group method with arithmetic averaging.

vitro propagated plants. Several DNA markers have been successfully employed to assess the genomic stability in regenerated plants including those with no obvious phenotypic alternations (Rahman and Rajora, 2001). Among the markers, inter simple sequence repeat (ISSR) (Zietkiewicz et al., 1994) and random amplified polymorphic DNA (RAPD) (Williams et al., 1990) have been mostly favored.

ISSR, a PCR based genetic marker system circumvents the requirement for flanking sequence information and thus has found wide applicability in a variety of plants (Srivastava and Gupta, 2008). ISSR technique has successfully been used for the assessment of genetic fidelity in *Robina ambigua* (Guo et al., 2006b) and medicinal herb *Swertia chirayita* (Joshi and Dhawan, 2007). ISSR technique has also been used to study genetic diversity in wild populations of endangered medicinal plant *Emmenopterys henryi* (Li and Jin, 2008) and a perennial herb *Glycyrrhiza uralensis* (Yao et al., 2008). The present communication documents the use of ISSR markers to assess the genetic similarity in tissue culture derived plantlets of *O. missionis.* In order to confirm genetic integrity, the DNA of 21 regenerated plants was compared to the DNA of single mother plant.

MATERIALS AND METHODS

Micropropagation

Tender branches were excised from 8-10 year old mature trees growing along the river bank side of Seetha nadhi in Udupi district. Multiple shoots were initiated from nodal explants on MS medium incorporated with 2.0 mg/l 6-benzylaminopurine (BA) and 0.3% (w/v) activated charcoal (AC). After 5 weeks, *in vitro* developed shoots were subcultured onto MS medium with BA (0.5 mg/l) and NAA (1.0 mg/l) and AC (0.3%) for shoot elongation. For *ex vitro* rooting, the base of shoots was dipped in different concentrations of IBA solution for different time duration and immediately transferred into plastic pots containing sterile soil-rite and acclimatized in growthchamber for one month. Then they were hardened in greenhouse for a period of 3-4 months.

Genomic DNA isolation

For ISSR analysis, fresh young leaf samples collected from the 21-tagged regenerated plants at the stage of hardening along with single mother plant for screening their genetic integrity. Total DNA was extracted using cetyl trimethyl ammonium bromide (CTAB) method described by Doyle and Doyle (1990) with minor modifications. Quality and quantity of DNA were inspected by both gel electrophoresis and spectrometric assays using UV-Visible Double Beam PC Scanning spectrophotometer (LABOMED Inc. Culver city, USA).

ISSR-PCR amplification

Polymerase chain reaction (PCR) amplification was performed with a total of 32 ISSR primers (Bangalore Genei, Bangalore, India). For each amplification, the reactions were carried out in a volume of 20 µl comprising 1.5 unit of *Taq* DNA polymerase (Bangalore Genei, Bangalore, India), 1x *Taq* assay buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl and 0.01% gelatin, pH 9.0), 200 µM of primer, 250 µM of dNTPs and 50 ng DNA template. Amplification was performed in thermocycler, UNO II (Biometra, Goettingen, Germany). The optimized PCR condition for ISSR analysis were consisted of an initial denaturation of 3 min at 94°C, followed by 40 cycles for 1 min at 94°C for denaturation, 1 min at annealing temperature, 1 min at 72°C for extension step. The final extension step was done for 10 min at 72°C and the reactions were kept at 4°C. The annealing temperature was dependent on the primers used.

The amplified products were separated by electrophoresis on 1.5% (w/v) agarose (Amersham Uppsala, Sweden) gels for 75-100 Volts in 1x TBE buffer (Tris–Borate–EDTA buffer). After completion of electrophoresis, gels were stained with ethidium bromide solution. The amplified products in gels were visualized and photographed using gel documentation Bioprofile Image Analysis System (Vilber Lourmat, France). The size of the amplification products were determined by comparison to λ DNA/*Eco*R I -*Hind* III Double digest DNA ladder (Bangalore Genei, Bangalore, India). PCR reactions were repeated at least twice to check reproducibility of the clear banding patterns.

Data scoring

PCR amplified clear and reproducible bands in the size range of 200 to 21,226 bp were scored with all the selected ISSR primers. The bands were transformed into a binary character matrix, “1” for presence and “0” for absence of band in each plant at a particular position, which was treated as an independent character regardless of its intensity. Data analysis was performed using the NTSYS-pc (Numerical Taxonomy System), version 2.1 computer program package (Rohlf, 2000). Genetic similarities between micropropagated and mother plants were used to calculate the Jaccard’s similarity coefficient (Jaccard, 1908). The similarity coefficients thus generated were used for constructing dendrogram using the UPGMA (unweighted pair-group method with arithmetic average) and the SHAN (Sequential hierarchical agglomerative nested clustering) option in NTSYS-pc software package.

RESULTS AND DISCUSSION

The assessment of the genetic stability of *in vitro* derived clones is an essential step in the application of biotechnology for micropropagation of true-to-type clones (Eshraghi et al., 2005). *In vitro* germplasm conservation relies on micropropagation methods. However, phenoltypic and genetic variations are reported to occur during in *vitro* regeneration processes, originating somaclonal variants (Kaeppler et al., 2000). Thus, the risks of genetic changes induced by tissue culture and the importance of assessing the genetic stability of the biological material along all phases of storage must be considered in the context of conservation.

Since somaclonal variation is expected under tissue culture conditions, the genetic fidelity of the shoots formed and the protocol developed for mass propagation need to be ascertained. To our knowledge, no report was available on the comparative genetic stability of regenerants and mother plant of *O. missionis* by using ISSR markers. In this paper, the micropropagation protocol standardized by Dalal and Rai (2001) was used to esta-

Table 1. ISSR primers used to screen twenty one micropropagated *O. missionis* plantlets.

Primers	Primer Sequences (5' to 3')	Annealing temp. Tm (ºC)	Number of bands		
			Polymorphic	Monomorphic	Total
ISSR02	CTCTCTCTCTCTCTCTAC	40	-	5	5
ISSR03	CTCTCTCTCTCTCTCTGC	40	-	9	9
ISSR04	CACACACACACAAC	45	-	4	4
ISSR05	CACACACACACAGT	45	-	5	5
ISSR06	CACACACACACAAG	45	1	7	8
ISSR07	CACACACACACAGC	45	-	5	5
ISSR09	GTGTGTGTGTGTGG	37	-	2	2
ISSR10	GAGAGAGAGAGACC	48	-	3	3
ISSR12	CACCACCACGC	32	-	11	11
ISSR13	GAGGAGGAGGC	32	-	5	5
ISSR14	CTCCTCCTCGC	45	1	3	4
ISSR15	GTGGTGGTGGC	32	-	6	6
ISSR16	GAGAGAGAGAGAGAGAGAT	48	-	9	9
ISSR19	GACAGACAGACAGACA	40	-	5	5
ISSR21	GATAGATAGATAGATA	32	-	6	6
ISSR22	CCTACCTACCTACCTA	40	1	5	6
ISSR01/W3	ACACACACACACACACC	45	-	9	9
ISSR02/W4	ACACACACACACACACG	45	-	8	8
ISSR04/W7	GGGTGGGGTGGGGTG	45	-	7	7
ISSR05/W8	CTCTCTCTCTCTCTCTG	45	-	8	8
ISSR06/W9	CACACACACACACACAG	45	-	6	6
ISSR07/W11	TCTCTCTCTCTCTCTCA	45	-	9	9
ISSR09/W30	GGAGAGGAGAGGAGA	45	-	6	6
ISSR10/15	CCCGTGTGTGTGTGT	45	-	4	4
ISSR11/20	CCAGTGGTGGTGGTG	45	-	8	8
ISSR12/32	AGAGAGAGAGAGAGAGC	45	-	6	6
ISSR13/33	GAGAGAGAGAGAGAGAT	45	2	4	6
ISSR14/34	GAGAGAGAGAGAGAGAC	45	-	6	6
ISSR15/8082	CTCTCTCTCTCTCTCTCTG	45	-	7	7
Total 29			5	178	183

blish the large scale *in vitro* plants. The ISSR marker technique was used to assess the genetic stability of micropropagated plants. The micropropagated plants were found to be phenotypically normal and essentially identical with their mother plant at hardening stage which partly suggest the minimal or absence of somaclonal variations. The present study confirms the fact that axillary multiplication is the safest mode of micro-propagation to produce true to type progeny. There are many reports in literature reporting similar results (Carvalho et al., 2004; Martins et al., 2004; Joshi and Dhawan, 2007).

Fingerprinting profiles of the culture regenerants and the donor plant were generated using a total of 32 ISSR primers, of which 29 primers produced distinct, reproducibly amplified products. Indeed, all these 29 primers were found to generate identical banding patterns in two independent amplifications that were performed for all the samples. A total of 183 amplification products were detected. The primer pairs amplified between 2 to 11 DNA fragments with an average of 6.31 fragments per primer. Among the 29 primers responded, 25 primers produced monomorphic patterns, while rest 4 primers generated polymorphic bands, at least in one of 21 individuals relative to the donor plant. Information on the selected ISSR primers, total number of bands scored, monomorphic bands and polymorphic bands for each primer are sumarized in Table 1. An example of monomorphic gel obtained in primer ISSR-04 is shown in Figure 1.

Of the total 183 bands scored, 178 bands produced were monomorphic (97.2%) and five bands were polymorphic (2.73%). On the similar note, low genetic variation of 3.92% among the 21 *in vitro* grown plants of *Dictyospermum ovalifolium* was reported (Chandrika et al., 2008). And about 97% homology between the mother plants and micropropagated plants has been reported in an endemic and endangered plant, *Syzygium travancorium* (Anand, 2003). However, Guo et al. (2006a) reported 15.7% of polymorphic bands in the ISSR analysis

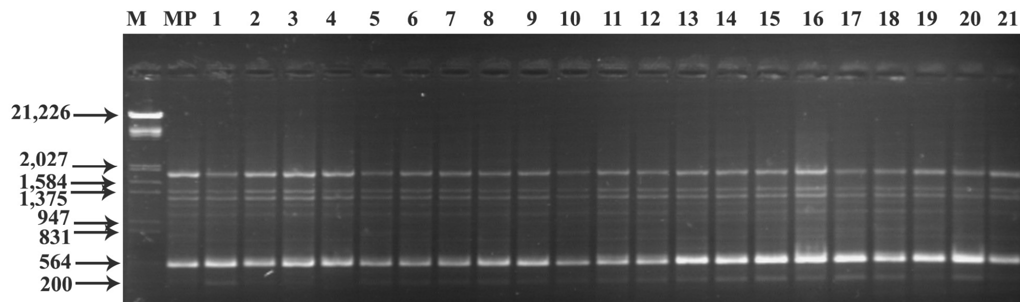

Figure 1. ISSR products generated from 21 *in vitro* regenerated plants and mother plant of *O. missionis* amplified with primer ISSR-04 shows monomorphic pattern. Lanes: M: Molecular size marker (λDNA/*Eco*RI- *Hind*III Double digest DNA ladder), MP: donor plant, 1-21 micropropagated plants.

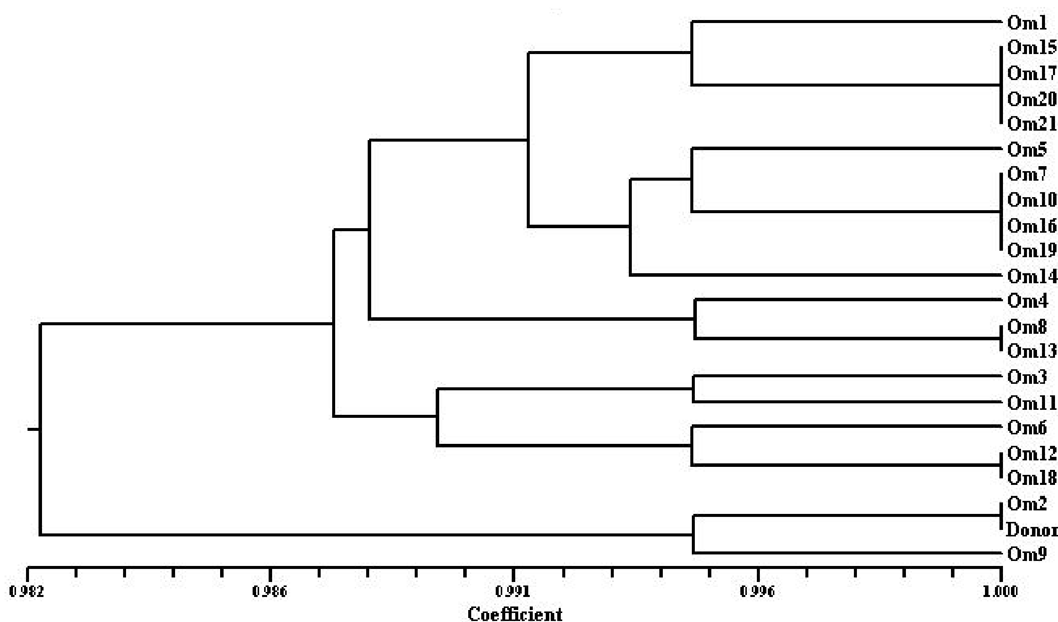

Figure 2. Dendrogram of twenty one hardening plants and the mother plant of *O. missionis* generated by UPGMA cluster analysis, based upon 183 ISSR bands depicting the genetic similarity between the individual plants. They were grouped into four clusters.

for the 63 regenerants of medicinal plant *Codonopsis lanceolata*. The genetic fidelity of micropropagated plants is often questioned because there are frequent reports on the occurrence of somaclonal variations (Martin et al., 2006; Ray et al., 2006).

In the present investigation, out of total 32 ISSR primers screened, 29 primers gave clear bands of 183 bands for 22 individuals (21 regenerated plants and 1 donor mother plant). A total number of 4,026 bands [numbers of total plantlets analyzed (22) * number of bands obtained from ISSR primers (183 bands)] were generated in all the plantlets analyzed. Similarly, a total number of 4,824 bands were obtained from the shoots of *Pinus thunbergii* (Goto et al., 1998), 5,088 bands in micropropagated shoots of banana (Venkatachalam et al., 2007), 925 bands in tissue culture clones of *Mucuna pruriens* (Sathyanarayana et al., 2008) and 7,172 total bands were generated in micropropagated almond plantlets exhibiting homogenous in both RAPD and ISSR patterns (Martins et al., 2004).

A dendrogram constructed on the basis of Jaccard's similarity matrix, followed by UPGMA based clustering analysis (Figure 2) showed that the genotypes were grouped into four clusters. Cluster I consisting of 11 tissue cultured-progenies among which two sub-clusters were formed. Subcluster Ia consists of Om15, Om17, Om20, Om21 and Om1 whereas subcluster Ib contains Om7, Om10, Om16, Om19, Om14 and Om5 with great resemblance among themselves showing 100% similarity level. Cluster II showed similarity between three (Om8,

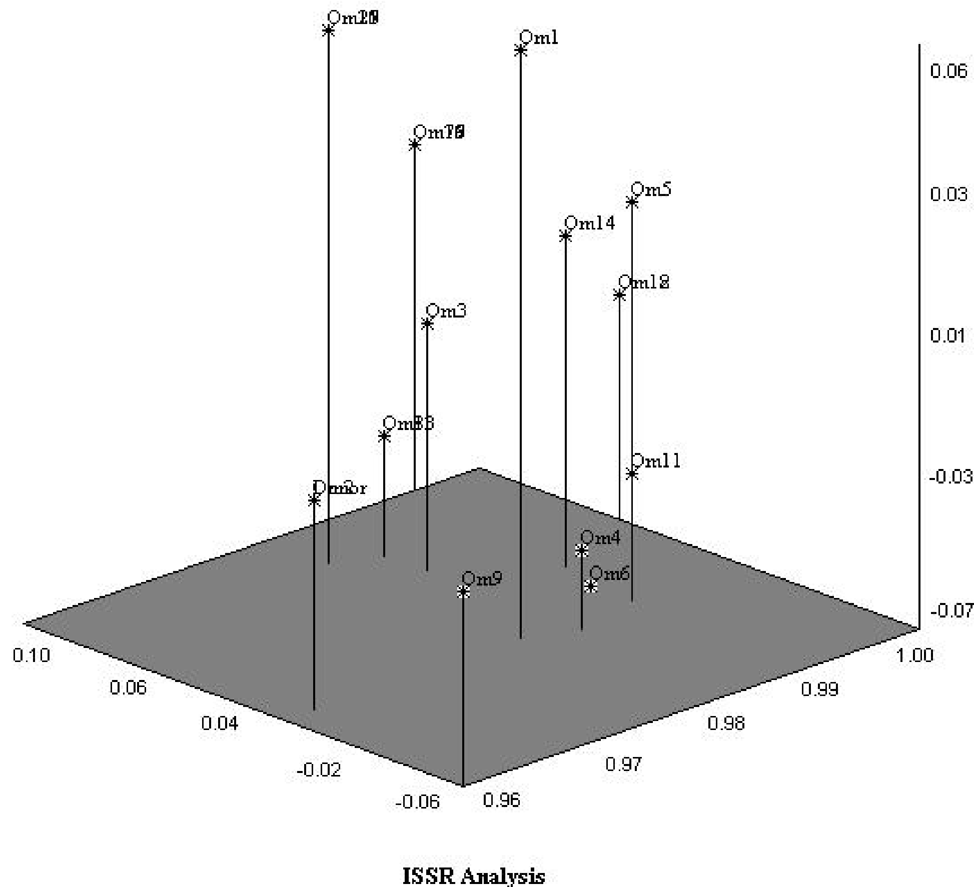

Figure 3. Principal coordinate analysis (PCA) using ISSR primer efficient similarities among 21 regenerated plants (Om-1 to Om-21) and the single donor plant of *O. missionis* by the UPGMA cluster analysis (NTSYS-pc).

Om13 and Om4 were similar) tissue cultured plants. There were five *in vitro* plants in cluster III exhibiting self genetic resemblance in Om3, Om11, Om6 however Om12 and Om18 showed 100% similarity. In Cluster IV, micropropagated plant Om2 and donor plant was grouped together along with Om9 progeny showing genetic fidelity. All three groups which are depicted separately shows they all come under one single donor plant in the fourth group, which shows similarity. Figure 3 shows the result of the principal coordinate analysis (PCA) among the 21 regenerated plants (Om-1 to Om-21) and the single donor plant.

The ISSR data were used to calculate genetic similarity among the 22 individuals (21 micropropagated plants and one donor plant) in a pairwise manner. The similarity coefficient among the plants ranged from 0.985 to 0.997 with a mean of 0.994. The dendrogram, generated by cluster analysis indicated 99% similarity. On the similar study in *Codonopsis lanceolata* using ISSR analysis, similarity coefficient of dendrogram among the 64 individuals ranged form 0.595 to 1.000 with a mean of 0.933 clustered into four groups with the donor plant in the second group showing 93% similarity (Guo et al., 2006a). Adetula (2006) generated dendrogram using UPGMA which was divided into four major clusters, showing 90% similarity level in *Capsicum* by RAPD analysis. Whereas in *Robinia pseudoacacia*, similarity indices ranged from 0.86 to 0.96 among eighteen micropropagated plants which grouped into one major cluster by RAPD at 86% similarity level, however, eight different clusters could be obtained at 90% similarity level (Bindiya and Kanwar, 2003).

From the present study, it has been concluded that ISSR analysis can be used to check the genetic consistencies in the micropropagated plants and donor plant of *O. missionis*. This medicinal tree species requires immediate attention with greater emphasis for habitat protecttion and incorporation of larger germplasm collection and other propagation techniques. *In vitro* plant regeneration and reintroduction of the threatened plants into the originnal or favorable habitats is one strategy for conservation of important medicinal plant species. The observation of low level polymorphism between genotypes supports the conclusion that these plantlets regenerated by the

method used in this study are likely to be genetically true to their trees of origin.

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