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Assessment of genetic fidelity in microclones of curry leaf plants [*Murraya koenigii* (L.) Spreng.] using ISSR markers

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Murraya koenigii (L.) Spreng., commonly called Indian curry leaf, is an aromatic shrub highly valued for its medicinal potentialviz. anticonvulsant, antitumor, anti-inflammatory, antidiabetic, antiviral and diuretic. Its leaves, locally known as curry patta, are used widely for culinary purpose, particularly in India. However, *M. koenigii*, due to poor rate of fruit set and seeds with short viability period and poor rate of germination, are sparsely distributed in wild, and has attracted researchers for propagation through biotechnological approaches. In this context, maintaining genetic fidelity is a vital for assessing genetic uniformity in micropropagated plantlets as variations within the progeny can result in serious losses to the end users. Therefore, it is necessary to screen them for their genetic makeup whether they are true-to-type or not. Molecular techniques like Inter Simple Sequence Repeat (ISSR) that are not influenced by environmental factors are appropriate tools to analyse genetic fidelity of *in vitro* propagated plants as, and generate reliable and reproducible results. In this study, we tried to evaluate genetic fidelity of micro-clones of tissue culture raised *M. koenigii* using ISSR technique. Twenty five ISSR primers were used to amplify genomic DNA from *in vitro* raised field grown plants and mother plant. Out of 25 primers screened, a total 465 amplified products were obtained from 10 ISSR primers. Out of which, 55 were monomorphic across the mother plant and its micropropagated progenies of 10 ISSR primers 05 showed profiles identical to mother plant. Similarity matrix based on Jaccard's coefficient and pair-wise values between mother plant and tissue cultured plant ranged from 0.91 to 1.00, indicating a high degree of genetic fidelity.

Keywords: Culinary, Indian curry leaf, Indian spices, Molecular markers, Traditional medicine

Murraya koenigii (L.) Spreng. (Fam.: Rutaceae), commonly known as Indian curry leaf tree, is a native of the Indian subcontinent. It shares aromatic nature, more or less deciduous shrub or tree up to 3-5 meters in height and 15 to 40cmin diameter with short trunk, thin smooth grey or brown bark and dense shady crown, and are used in the traditional system of medicine in Eastern Asia[1,2]. The *Murraya* species is the richest source of carbazole alkaloids, reported for their various pharmacological activities such as anticonvulsant, antitumor, anti-inflammatory, diuretic, antiviral and activities[3]. *Murraya* leaves are full of antioxidants, namely, tocopherol, β-carotene, and lutein, and possess antioxidative activities, providing protection against oxidative stress[4,5]. Koenidine, a metabolically stable carbazole alkaloid isolated from the leaves of *M. Koenigii* demonstrated a considerable reduction in the postprandial blood glucose level and improved insulin sensitivity in streptozotocin-induced diabetic rats[6].

In order to cater the increasing demands of herbal drug market, conservation and commercial production of these plants have become necessary. Since, conventional methods of propagation are generally slow, labour intensive, requiring large number of propagules and limited success, modern methods of biotechnology have taken lead in propagation of plants[7,8]. Consequently, micropropagation has now become an important thrust area for medicinal plant research[9-12]. Clonal propagation and preservation of elite genotypes, selected by their superior characteristics, require high degree of genetic uniformity among the regenerated plants. In medicinal plants, it is all the more important to ensure that there is no variation in the amount and quality of active principles. Tissue culture is an efficient method of clonal propagation; however, the resulting regenerants often has a number of somaclonal variations[13]. Occurrence of somaclonal variation is a disadvantage

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for both *in vitro* cloning as well as germplasm preservation method, and therefore, the investigation of genetic variability/stability of *in vitro* plants is extremely important.

Among various pathways of micropropagation enhanced axillary, adventitious branching, somatic embryogenesis is generally considered to be immune to genetic changes that might occur during cell division or differentiation[14,15]. There are several cases where genetic stability has been demonstrated in adventitious shoot bud, callus and cell suspension culture regenerants[16-18]. However, there are several examples where instability has been reported in plants regenerated from highly organized state of shoot apex and meristem cultures[19,20].

DNA based molecular markers are reliable source to access the genetic variability of *in vitro* regenerated plants. These are not influenced by environmental factors, and hence generate reliable and reproducible results. ISSR markers comprise a few highly informative multi-allelic loci. They provide highly discriminating information with good reproducibility, and are relatively abundant[21,22]. Inter simple sequence repeat (ISSR) has been mostly favoured and successfully applied to detect genetic similarities or differences in tissue-cultured materials of various plants. Since ISSR markers amplify different regions of the genome, their use allows better analysis of genetic fidelity/stability of the plantlets[23-26].

ISSR analysis was effective to eliminate the somaclonal variant in *in vitro* leaf-derived horseradish plants[27]. A few informations are available on genetic relationships among wild and cultivated accession in *M. Koenigii*[28] and its relatives[29] but as such no reports are available on the molecular evaluation of genetic fidelity in micropropagated plants of *M. koenigii* using ISSR markers. Hence, in this study, we have made an attempt to evaluate genetic fidelity of micropropagated plants of *Murraya koenigii* produced axillary bud, inter-node explants from approximately 10-year old matured plant and leaf, cotyledons, cotyledonary node (embryonic axis), hypocotyls and root segments from *in vitro* raised seedlings[30] using ISSR markers.

Material and Methods

Genomic DNA extraction and PCR amplification

Regenerants developed from all the seven type of explants (may be mentioned again here for clarity) were selected randomly for genetic fidelity assessment. Genomic DNA from leaves of regenerants and mother plant (P) was extracted using CTAB method[31]. The total genomic DNA was quantified spectro-photometrically (UV-Vis Spectrophotometer, Pharmaspec UV-1700, Shimadzu, Japan) and aliquots were diluted to the final concentration of 10-15 ng μL^{-1}. PCR reaction for ISSR was performed in a programmable thermal cycler (Master cycle epgradient S, Eppendroff, Germany).A total of 25 ISSR primers of 800P and UBC series (Eurofins Genomics, India) were initially screened for their applicability in PCR amplification of total genomic DNA of *M. koenigii*. Out of 25 ISSR, only 10 produced clear and reproducible amplified products.

ISSR profiles were produced through PCR amplification using the protocol described by Verma & Rana[28]. PCR amplification was carried out in 25 μL volume using 10 different decamer primers. The reaction buffer consisted of 2.5 μL of 10X PCR buffer, 2.0 μL $MgCl_2$ (2.5 mM), 0.50 μL dNTPs (10 mM each of dATP, dGTP, dTTP and dCTP) (Bangalore Genei, India), 2 μL primer, 0.2 μL DNA Taq polymerase (*In vitrogen* platinum), 5 μLDNA sample and 12.80 μL water. The primers showing polymorphic bands were then used for analysing the clonal fidelity of micro-propagated plants.

For all the samples, PCR programme involved an initial denaturation at 94°C for 04 min followed by 35 cycles of 1.0 min denaturation at 94°C, 1.0 min primer annealing at 52°C, 2 min primer extension at 72°C and final extension for 7 min at 72°C. The amplified PCR-ISSR products were electrophoresed in 1.5% agarose in 1X TBE (90 mM Tris-borate, 2 mM EDTA, pH 8.0) buffer. The gels were stained with ethidium bromide and documented using gel documentation system (Expert Vision, Mumbai, India).

Data analysis

For *in vitro* plantlet regeneration, all the experiments were set up in a Randomized Block Design (RBD). Each experiment was repeated thrice with minimum 10 replicates per treatment. The data were analyzed statistically using SPSS ver. 7.5 (SPSS Inc., Chicago, USA) and the results are express as means ± SD[32].For genetic fidelity analysis, the fingerprints were scored considering fragment size at a locus as bi-allelic (present = 1, absent = 0). Reactions with each primer were repeated at least thrice and only those fragments

that were well resolved and reproduced in each instance were scored and included in the analysis ignoring the bands intensity. ISSR profiles obtained for *in vitro* derived regenerants were compared with mother plant.

Results and Discussion

Genetic fidelity assessment using ISSR primers

In this study, ISSR marker were used to characterize and analyze the genetic fidelity between mother plant and micropropagated plants of *M. koenigii* were also analyzed using 25 ISSR primers of which 10 generated amplified products. Out of 10 ISSR markers tested 05 produced reproducible polymorphic banding patterns. In 800 P series of ISSR primers, 811 P and 826 P primers didn't show any polymorphism and they generated 40 and 60 loci with band range size between 250 and 850 bp, and 100 and 1500 bp, respectively. Primer 818 P and 815 P produced 63 and 60 bands with 12.5 and 22.22% polymorphism respectively (Fig. 1 A-B).

Total five ISSR primers of UBC series were tested out of which three didn't generate polymorphic bands. In UBC series of ISSR primers, the highest percentage of polymorphism 50% with a 24 amplified bands were generated by UBC 857 primer in which the band size was ranging between 400 and 1000bp (Table 1), which in followed by 815 P (22.22%) > 814 P (20%) > UBC 881 (14.29%) and lowest percentage of polymorphism was observed in 818 P (12.50%). Hence, the observations of present study on the basis of ISSR, fingerprinting patterns resulted that the micropropagated plant of *Murraya koenigii* showed genetic fidelity with their mother plant.

Similarity coefficient Analysis of ISSR Profiles

Jaccard's pair-wise similarity coefficient values among micropropagated and mother plants of *M. koenigii* were ranged from 0.91 to 1.00 (Table 2). In our study, 91% similarity coefficient was observed in maximum number of samples screened. The highest similarity (98%) was observed between P^1-P^5, P^1-P^6, P^5-M and P^6-M, while the lowest value 0.91 was obtained between P^1-P^2, P^1-P^4 and P^4-M.

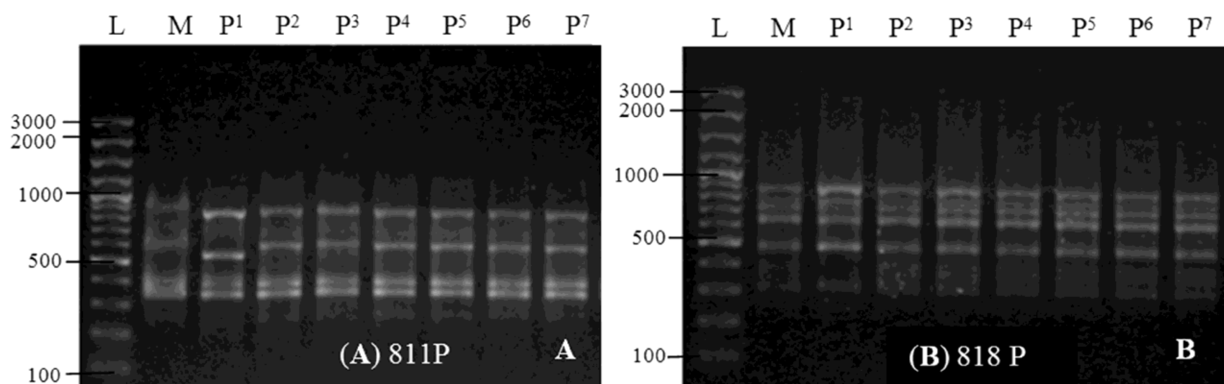

Fig. 1 — Fingerprinting profile of mother plant (M) and micropropagated plants via different pathways (P^1 Inter-nodal, P^2 Leaf, P^3 Root, P^4 Cotyledon, P^5Cotyledonary Node, P^6Hypocotyl, P^7 nodal (Axillary bud) explant) of *Murraya koenigii*, generated by ISSR primers (A) 811P; and (B) 818 P. [L represents 100bp ladder]

Table 1 — Total number of amplified and polymorphic loci generated by selected 10 ISSR primers in micropropagated and mother plant of *Murraya koenigii*

Primer name	Sequence 5′–3′	Tm (℃)	Polymorphic Loci	Monomorphic Loci	Percentage polymorphism	Loci amplified	Approx. band range size (bp)
811P	(GA)$_8$C	52.8	00	06	00.00	48	250-850
814P	(CT)$_8$ A	50.4	01	04	20.00	33	300-900
815P	(CT)$_8$G	52.8	02	07	22.22	60	350-1,750
818P	(CA)$_8$G	52.8	01	07	12.50	63	300-850
826P	(AC)8C	52.8	00	05	00.00	40	100-1.500
UBC 810	(GA)8T	50.4	00	04	00.00	32	250-1,000
UBC 842	(GA)8YG	54.8	00	07	00.00	56	200-1,500
UBC 857	(AC)8YG	54.8	02	02	50.00	24	400-1,000
UBC 880	(GGAGA)3	50.6	00	07	00.00	56	250-1,000
UBC 881	(GGGTG)3	58.8	01	06	14.29	53	500-2,000
		Total	07	55	11.90	465	

Table 2 — Jaccard's similarity coefficient of micropropagated and mother plants of *Murraya koenigii* by ISSR primers

	P1	P2	P3	P4	P5	P6	P7	M
P1	1.00							
P2	0.91	1.00						
P3	0.93	0.95	1.00					
P4	0.91	1.00	0.95	1.00				
P5	0.98	0.93	0.94	0.93	1.00			
P6	0.98	0.93	0.94	0.93	1.00	1.00		
P7	0.93	0.95	0.96	0.95	0.94	0.94	1.00	
M	0.96	0.91	0.93	0.91	0.98	0.98	0.96	1.00

[(M) Mother plant, P1 - P7 micropropagated plants regenerated via different pathways (P1 Inter-nodal, P2 Leaf, P3 Root, P4 Cotyledon, P5Cotyledonary Node, P6Hypocotyl, P7 nodal (axillary bud) explant) of plant tissue culture]

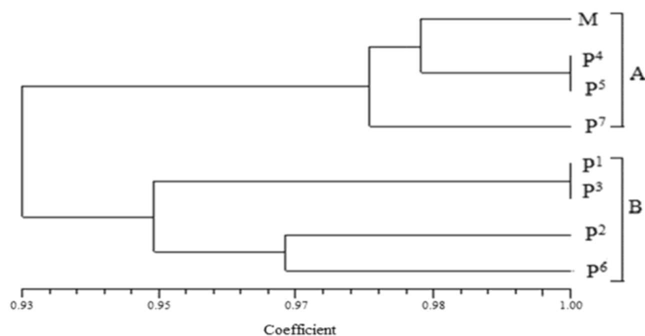

Fig. 2 — UPGMA dendrogram of micropropagated and mother plants of *Murraya koenigii* by ISSR primers

Cluster analysis of ISSR data

The cluster constructed through NTSYS-pc presented in the form of dendrogram (Fig. 2) which illustrated the overall genetic fidelity or relationship among micropropagated and mother plants of *M. koenigii* patterns generated by 10 ISSR primers. The dendrogram was all the plants in two major cluster groups (group A and group B). Group A comprises of four plants M, P4, P5 and P7 which was further differentiated into two sub cluster, while group B included four plants (P1, P2 P3 andP6). Molecular markers have been used successfully to determine the degree of relatedness among individuals or group of accessions to clarify the genetic structure or variation among accessions, population, varieties and species[24,33].

ISSR markers can be used in population genetic studies of plant species as they effectively detect very low levels of genetic variation[34]. On the basis of number, intensity and reproducibility of ISSR bands, 10 primers were selected out of the 25 primers tested in *M. koenigii.* The banding pattern of PCR amplified product from micropropagated plantlets was found to be monomorphic for most of the primer tested. Bands with same mobility were considered as identical irrespective of their band intensity.

Molecular markers have been utilized for detecting variation or confirmation of genetic fidelity during micropropagation[35]. ISSR primer based assessment of regenerants has reported already in *Cornus alba*, *Cucumis melo* L., *Rauwolfia tetraphylla* L., *Simmondsia chinensis*, *Spilanthes calva*, *Tylophora indica* and *Zea mays*[36-42].The present report could be possibly the first report in which the comparative genetic fidelity analysis of regenerants are developed using all types of explants and mother plants of *M. koenigii* using ISSR markers.

Genetic uniformity is one of the most important prerequisites for successful micropropagation of any plant species. Nevertheless, a major problem encountered in cells grown *in vitro* is the occurrence of genetic variation. Although the origin of variation is unclear, it points to two main factors; an intrinsic factor, while largely depends on the genetic fidelity of the explants and an intrinsic factor, depending on culture media and particularly PGRs[43].

The ISSR technique has been used successfully for analyzing the genetic fidelity of species propagated through *in vitro* shoot formation[44,45]. Dendrogram was constructed on the basis of Jaccard's similarity matrix, followed by UPGMA based clustering analysis of ISSR profiles. The dendrogram showed the genotype of micropropagated and mother plant of *Murraya koenigii* were grouped into two major clusters A and B. Cluster A comprises with 3 groups and B with 3 groups. The ISSR data were also used to calculate genetic similarity between seven samples of micropropagated and mother plant of *Murraya koenigii* in pair-wise manner.

Conclusion

In this study, the true to the type nature of the *in vitro* raised micro-clones of *Murraya koenigii* was confirmed using ISSR markers. No variability was detected among the tissue culture-raised plantlets; hence, nodal explants can be successfully employed for the commercial multiplication of *Murraya koenigii* without much risk of genetic instability.

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Conflicts of interest

Authors declare that they have no conflict of interest.

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