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RAPD Markers for Genetic Analysis in Micropropagated Plants of *Dictyospermum ovalifolium*, a Rare Plant of Western Ghats, India

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ABSTRACT

Random amplified polymorphic DNA (RAPD) was used to assess the genetic fidelity of tissue-cultured *Dictyospermum ovalifolium* plants in relation to their mother plant. Thirty eight RAPD primers were screened, out of which 33 generated a total of 216 clear and reproducible bands. Of these, 202 were monomorphic and showed 93.5% clonal fidelity while 14 bands showed 6.45% polymorphism. The amplification products were monomorphic across all micropropagated plants with a low level of variation. A total of 4752 bands were generated showing uniform banding patterns. The genetic coefficient based on UPGMA showed 97% similarity between mother plant and micropropagated plants. The results confirmed the clonal fidelity of tissue-cultured plantlets and the fact that axillary shoot multiplication is the one of the safest modes for multiplication of true-to-type plants.

Keywords: conservation, dendrogram, genetic stability, medicinal plant, reintroduction
Abbreviations: CTAB, cetyl trimethylammonium bromide; **PCR**, polymerase chain reaction; **RAPD**, random amplified polymorphic DNA; **SHAN**, sequential hierarchical agglomerative nested clustering; **UPGMA**, unweighted pair group method with arithmetic averaging

INTRODUCTION

Dictyospermum ovalifolium (Commelinaceae) is a rare medicinal herb endemic to the Hulical Ghat region of Western Ghats, Karnataka, India (Nayar and Sastry 1988). It grows along the margins of evergreen forests with other herbaceous plants in moist humus-covered soil. It is one of the very few endemic plants which can also be found in the Western slopes of Niligiri hills. *D. ovalifolium* has gained considerable interest for its horticultural and medicinal importance, as its long roots are used in the treatment of allergies and skin diseases (Anonymous 2005). The clearing of forests with natural habitat loss has resulted in the depletion of natural populations. This in turn, has lead to increased interest in the *in vitro* propagation of these plants as a means for their mass propagation and conservation. In order to utilize the advantages of micropropagation, true-to-type reproduction is highly desirable. However, *in vitro* culture is mutagenic and DNA variation exists, i.e. somaclonal variation (Larkin and Scowcroft 1981).

In recent years, several DNA markers have been successfully employed to assess the genomic stability in regenerated plants, including those with no obvious phenotypic alternations. The most frequently used PCR-based DNA markers are random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR). The main advantages of RAPD are suitability for work on anonymous genomes and only a limited quantity of DNA is required. RAPD analysis is particularly well suited to high-output systems required for plants because it is easy to perform, fast, reliable and relatively low in cost (Williams *et al.* 1990). RAPD markers have been useful in establishing the genetic stability of *in vitro* regenerated plants of *Robinia pseudoacacia* (Bindiya and Kanwar 2003), *Swertia chirata* (Chaudhuri *et al.* 2007), *Platanus occidentalis* (Sun *et al.* 2008), *Ochreinauclea missionis* (Chandrika and Rai 2009) and *Nothapodytes foetida* (Chandrika *et al.* 2010).

This is the first report to detect the true-to-type nature of micropropagated *D. ovalifolium* plants compared with the DNA profile of their mother plant by using RAPD markers. An efficient tissue culture protocol for mass propagation of *D. ovalifolium* through rapid axillary bud proliferation was reported by Thoyajaksha and Rai (2001). An attempt was made to reintroduce the *in vitro* grown micropropagated plants to their original habitat in Western Ghats, Karnataka, India (Chandrika *et al.* 2008).

MATERIALS AND METHODS

Micropropagation

Healthy young branches of *D. ovalifolium* were collected from a wild population near Hulical Ghats of the Western Ghat region in Karnataka, India. Nodal segments were surface sterilized and transferred to MS medium (Murashige and Skoog 1962) fortified with 3.0 mg/l 6-benzyladenine (BA) (Sigma Aldrich Chemicals Pvt. Ltd., Bangalore, India) for shoot induction and multiplication. Multiple shoots were rooted *in vitro* on MS medium supplemented with auxins indole-3-acetic acid (IAA) at 1.0 mg/l and indole-3-butryic acid (IBA) at 2.0 mg/l (Sigma Aldrich Chemicals Pvt. Ltd., Bangalore, India). The rooted plantlets were transplanted to plastic pots containing Soilrite® and kept in a growth chamber (Sanyo, Osaka, Japan) at 25 ± 2°C and irradiance of 50 µmol m^{-2} s^{-1} with a 16-h photoperiod and 80% relative humidity for acclimatization. Plantlets were hardened in a greenhouse for 3-4 months before checking their genetic stability.

DNA extraction

Twenty one-tagged regenerated plants from hardening stage were randomly selected along with single mother plant for screening their genetic integrity. Total DNA was extracted from fresh young

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Original Research Paper

Table 1 RAPD primers used to screen 21 micropropagated *Dictyospermum ovalifolium* plantlets.

Primers	Sequence (5' to 3')	Size range (bp)	AT (°C)	Number of bands		
				PB	MB	Total
OPA-04	AATCGGGCTG	1584-200	40.0	2	4	6
OPA-11	CAATCGCCGT	2027-947	40.0	0	4	4
OPA-14	TCTGTGCTGG	3530-200	35.9	3	9	12
OPA-20	GTTGCGATCC	2185-564	35.0	0	11	11
OPB-01	GTTTCGCTCC	1904-564	41.5	0	6	6
OPB-04	GGACTGGAGT	1904-831	39.6	0	5	5
OPC-01	TTCGAGCCAG	1850-831	35.0	2	5	7
OPC-02	GTGAGGCGTC	1584-200	33.0	2	6	8
OPC-04	CCGCATCTAC	1904-200	33.0	0	8	8
OPC-05	GATGACCGCC	1460-550	40.0	0	6	6
OPC-07	GTCCCGACGA	1650-564	40.0	0	8	8
OPC-08	TGGACCGGTG	1584-564	40.0	0	5	5
OPC-11	AAAGCTGCGG	1375-564	39.6	0	5	5
OPC-15	GACGGATCAG	1904-200	31.5	2	8	10
OPD-05	TGAGCGGACA	1370-500	36.9	0	5	5
OPD-06	ACCTGAACGG	2027-831	39.5	1	8	9
OPD-07	TTGGCACGGG	2027-200	45.0	0	8	8
OPD-09	CTCTGGAGAC	1865-947	39.6	0	4	4
OPD-11	AGCGCCATTG	1375-947	41.2	0	3	3
OPD-16	AGGGCGTAAG	2567-564	34.2	0	10	10
OPE-03	CCAGATGCAC	1686-820	36.9	0	5	5
OPE-05	TCAGGGAGGT	1584-200	31.5	2	7	9
OPE-06	AAGACCCCTC	1402-564	39.6	0	5	5
OPE-19	ACGGCGTATG	1550-831	39.6	0	6	6
OPE-20	AACGGTGACC	1375-200	32.7	0	4	4
OPM-16	GTAACCAGCC	1904-831	45.0	0	5	5
OPM-20	AGGTCTTGGG	1765-564	40.0	0	4	4
OPN-03	GGTACTCCCC	2250-200	40.0	0	8	8
OPN-04	GACCGACCCA	1643-564	40.0	0	6	6
OPN-06	GAGACGCACA	1075-200	33.0	0	7	7
OPN-09	TGCCGGCTTG	1478-500	45.0	0	6	6
OPN-10	ACAACTGGGG	1267-200	33.0	0	5	5
OPN-12	CACAGACACC	3468-564	45.0	0	6	6
Total 33				14	202	216

AT-Annealing Temperature, **PB**-Polymorphic Bands, **MB**-Monomorphic Bands

leaves of micropropagated plants and field grown mother plant using cetyl trimethyl ammonium bromide (CTAB) method described by Doyle and Doyle (1990). Quantity of DNA was inspected by both gel electrophoresis and spectrometric assays using UV-Visible Double Beam PC Scanning spectrophotometer (LABOMED, Culver city, USA).

RAPD-PCR amplification

A total of 38 random decamer primers were obtained from Operon technologies (Sigma Aldrich Chemicals Pvt. Ltd., Bangalore, India) for PCR amplification. Of 38 RAPD primers screened initially, 33 were selected. Each reaction was carried out in a total volume of 20 µl reaction mixture containing 2.0 µl of 1X assay buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl and 0.01% gelatin, pH 9.0), 250 µM dNTPs, 200 µM primer, 1.5 U *Taq* DNA polymerase (Genei, Bangalore, India) and 50 ng DNA template. Amplification reactions were performed in an UNO II (Biometra, Goettingen, Germany) thermocycler, using a PCR program consisting of a 94°C initial denaturation for 3 min, followed by 40 cycles for 1 min at 94°C for denaturation, 1 min at annealing temperature, 2 min extension step at 72°C and a final extension step of 10 min at 72°C. The annealing temperature was adjusted according to the primers used in the PCR (**Table 1**).

Amplified products were separated on 1.5% (w/v) agarose gel (Amersham Uppsala, Sweden) using in 1X TBE buffer (Tris–Borate–EDTA buffer) at 75-100 V. The gels were stained with 3-5 µl of ethidium bromide, from 10X stock solution. The amplified products were visualized and photographed under UV transilluminator and documented using Bioprofile Image Analysis System (Vilber Lourmat, France). The size of the amplification products was estimated from lambda DNA/*Eco*RI-*Hind*III Double digest (Genei, Bangalore, India) as molecular marker. All PCR reactions were repeated twice to check the reproducibility of results.

Data analysis

PCR amplified bands in the size range of 200 to 21,226 bp were scored with all the selected RAPD primers. The bands were transformed into a binary format, "1" for the presence and "0" for the absence for each plant at a particular position. Genetic similarities between micropropagated and mother plants were measured by Jaccard's similarity coefficient (Jaccard 1908) with the SIMQUAL module. The similarity coefficients thus generated were used for constructing a dendrogram using the UPGMA and SHAN option in NTSys-pc version 2.1 software program package (Rohlf 2000).

RESULTS

Micropropagation

Previously standardized micropropagation protocol of *D. ovalifolium* was used to establish the large scale propagation of *in vitro* plants (Thoyajaksha and Rai 2001). By using MS medium fortified with 3.0 mg/l 6-benzyladenine, 58.3% of the nodal explants showed multiple shoot formation in about 4-5 weeks. At the same time, 2-3 repeated subculturing of nodal segments, at the intervals of 30 days from *in vitro* grown shoots helped to achieve continuous production of callus free healthy shoots. After being transferred onto the root induction MS medium supplemented with auxins indole-3-acetic acid (IAA) at 1.0 mg/l and indole-3-butryic acid (IBA) at 2.0 mg/l 91.7% of shoots, rooted into plantlets. After 2 months, more than 95% of the regenerated plants survived after being transferred into pots in growth chamber. Then plantlets were hardened in greenhouse conditions for 5-6 months before moving to its original habitat.

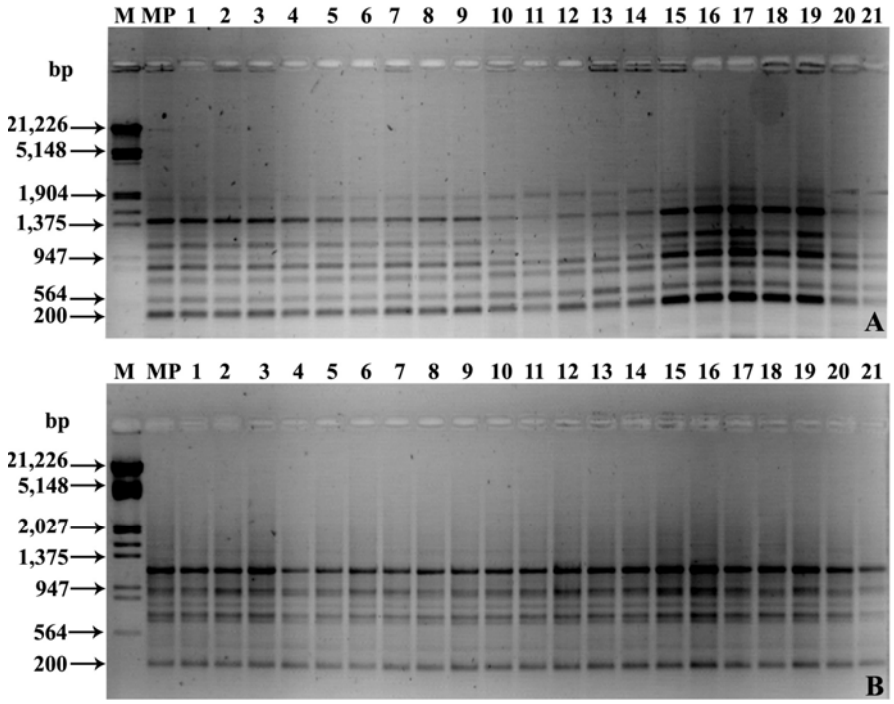

Fig. 1 Monomorphic amplifications produced in *D. ovalifolium* plantlets using RAPD primers (**A**) OPC-04 and (**B**) OPN-06.

Genetic stability by using RAPD markers

Of the total of 38 RAPD primers screened for the analysis, only 33 were useful in reproducing the patterns, so the remaining five primers were discarded as they produced ambiguous and non-reproducible amplification profiles. The remaining 33 primers produced a total of 216 clear bands 202 of which (93.5%) produced were monomorphic and 14 bands (6.45%) were polymorphic among the 22 individual plants (21 regenerants and one donor plant) which ranged from 200 to 21,226 bp in size. Detailed information on the selected RAPD primers, their annealing temperature, total number of bands scored, monomorphic and polymorphic bands for each primer are summarized in **Table 1**. The number of bands produced per primer ranged from 3 to 12 with an average of 6.54 bands per primer. In *D. ovalifolium* plantlets, a total of 4752 bands [total number of plantlets analyzed (22) × total number of bands scored from all primers (216)] were generated using RAPD primers giving rise to monomorphic patterns across all 22 plantlets analyzed. Monomorphic DNA profiles of *in vitro* regenerants were observed in RAPD primers OPC-04 and OPN-06 (**Fig. 1A, 1B**). A polymorphic gel is represented in primer OPA-04, which includes the loss of original bands (at approximately 1584 and 1265 bp, marked by arrows), as shown in **Fig. 2A**.

Data evaluation

The RAPD data were used to calculate genetic similarity among the 22 individuals (21 regenerated plants and 1 donor plant). A dendrogram was constructed on the basis of Jaccard's similarity matrix, followed by UPGMA-based clustering analysis (**Fig. 2B**) showed that the genotypes were grouped into one single major cluster within donor plant but Do-1 and Do-2 plant replicates showed significant changes. The coefficient of similarity in the dendrogram generated by the RAPD data among the regenerated plants ranged from 0.961 to 0.995 with a mean of 0.979. 97% of the plants were genetically similar.

DISCUSSION

One of the most crucial concerns in the *in vitro* propagation is to retain the genetic fidelity of the micropropagated plants with respect to the mother plant. Molecular markers have been used to detect somaclonal variation among the micropropagated plants (Palombi *et al.* 2007; Tawar *et al.* 2008; Sun *et al.* 2008; Ahmed *et al.* 2009). Screening the tissue culture derived plants using molecular markers will assist in reintroducing true-to-type plants (Heinze and Schmidt 1995) and protecting their genetic integrity. More recently two molecular markers like ISSR and RAPD have particularly been suggested to be useful for confirmation of genetic stability in micropropagated plants (Guo *et al.* 2006; Ray *et al.* 2006; Joshi and Dhawan 2007). There are several reports documenting observation as for somoclonal phenotypic variants in turmeric (Tyagi *et al.* 2007), *Hagenia abyssinica* (Feyissa *et al.* 2007) and *Platanus acerifolia* (Huang *et al.* 2009). RAPD technique has been used to detect somaclonal variation in sugarcane (Saini *et al.* 2004; Jain *et al.* 2005).

In the present investigation, out of 33 RAPD primers used, 216 clear bands were produced with 93.5% monomorphism and 6.45% polymorphism showing low level of genetic variation, indicating the stability of regenerated plants. Devarumath *et al.* (2007) reported RAPD analysis of plants propagated from micropropagated sugarcane which were genetically stable and showed clonal fidelity of >97%. Similarly, uniform monomorphic banding pattern has been reported earlier in various *in vitro* raised plants of *Tectona grandis* and chestnut hybrids using RAPD primers (Gangopadhyay *et al.* 2003; Carvalho *et al.* 2004). Whereas Ray *et al.* (2006) observed 39.28% polymorphism among three cultivars of banana. Bindiya and Kanwar (2003) reported 32% polymorphism in *Robinia pseudoacacia* and Modgil *et al.* (2005) achieved 23.2% polymorphism in micropropagated apple plants.

In *D. ovalifolium* plantlets, a total of 4752 bands were generated using RAPD primers with bands varying from 3 to 12, with an average of 6.54 bands per primer. On the other hand, a total of 925 scorable bands, which varied from 3 to 9, with an average of 6 bands per primer were reported from tissue culture clones of *Mucuna pruriens* (Sathyanarayana *et al*. 2008). 1056 bands were obtained from *Syzygium travancoricum* micropropagated plants which ranged from 2 to 8 with an average of 4.6 bands per primer (Anand 2003). Similarly, a total of 5590 bands were observed in micropropagated plants of *Curcuma longa* (Panda *et al.* 2007), 5472 bands were obtained in *Gossypium hirsutum*

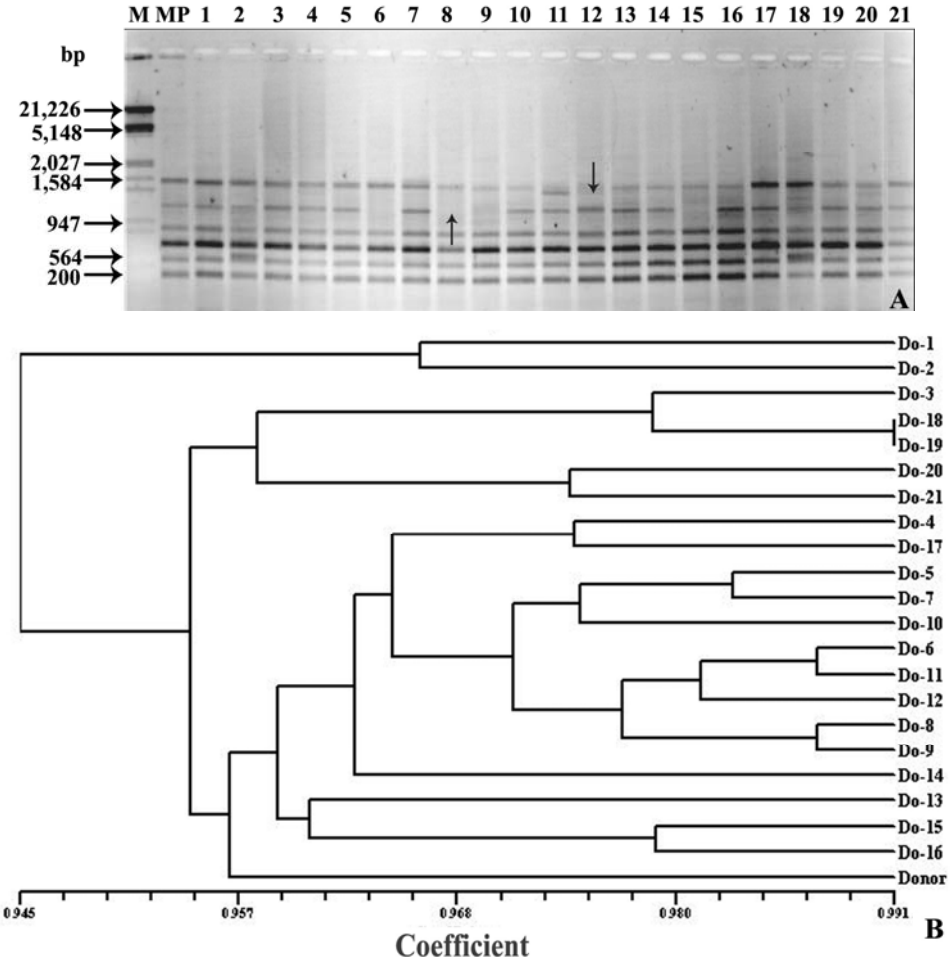

Fig. 2 RAPD polymorphic gel profile generated in *Dictyospermum ovalifolium* plantlets in a primer. (**A**) OPA-04 which includes loss of original bands (at approximate size of 1584 and 1265 bp, marked by arrows). (**B**) Dendrogram illustrating similarities among 21 regenerated plants (Do-1 to Do-21) and donor plant in *D. ovalifolium* plantlets using UPGMA cluster analysis based on RAPD markers. Lane M: Molecular size marker λ DNA/*Eco*RI-*Hind*III double digest DNA ladder. Lane MP: mother plant, Lanes 1-21: micropropagated plants.

(Jin *et al.* 2008) and 5390 bands were reported in micropropagated plants of *Ochreinauclea missionis* (Chandrika and Rai 2009) during RAPD analysis.

Reintroduction of *in vitro*-grown *D. ovalifolium* plants has been previously reported (Chandrika *et al.* 2008). The reintroduction program was carried out in two different regions of a reserve forest area near Hulical and Etthankatte of Western Ghats region, Karnataka, India in three phases. In the first phase 1000 micropropagated plants were successfully transplanted, followed by 1000 plantlets during the second phase and more than 500 plants in the third phase which were consequently reintroduced in the same area. The establishment and mortality rates were subsequently recorded for two years. Most of the *D. ovalifolium* plants reintroduced in areas of Hulical Ghat showed better establishment with a survival rate of 88% when compared to the Etthanakatte region with a survival rate of 40.8%.

CONCLUDING REMARKS

In the present study, a protocol for assessing the genetic stability of micropropagated plantlets and mother plant of *D. ovalifolium* by using RAPD markers has been achieved. In order to confirm genetic integrity, the DNA of 21 regenerated plants was compared with the DNA of the mother plant. A molecular analysis of plants at the hardening stage provides an early evaluation of plants for clonal fidelity and helps to save time and energy. We can conclude that the *in vitro*-grown *D. ovalifolium* plants developed in this study had a low level of 6.45% polymorphism and high level of 93.5% monomorphism.

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