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BRIEF COMMUNICATION

Assessment of genetic stability of *in vitro* grown *Dictyospermum ovalifolium*

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Abstract

In the present study, a polymerase chain reaction (PCR)-based method namely inter simple sequence repeat (ISSR) was employed to assess genetic stability in tissue culture-derived *Dictyospermum ovalifolium* plantlets. To study genomic stability of micropropagated plants, 14 individuals were randomly tagged among a population of 2500 regenerants and were compared with single donor mother plant. A total of 51 clear and reproducible bands ranging from 200 bp to 2.1 kb were scored corresponding to an average of 3.64 bands per primer. Two of the 51 bands were polymorphic (3.92 %) among 14 individuals, thus indicating the occurrence of low level genomic variation in the micropropagated plants. Cluster analysis indicates that genetic similarity values were 0.978 which allows classification of the plants to distinct groups. Further an attempt was made to reintroduce the micropropagated plants into their natural habitat. Over one thousand six hundred fifty plants were successfully established.

Additional key words: ISSR molecular marker, PCR, micropropagation, reintroduction, conservation.

The preservation of genetic diversity both within and among natural population is a fundamental goal of conservation biology. Biotechnological methods are an essential component of plant genetic resources management (Benson 2000) and they are becoming increasingly important for the conservation of rare and endangered plants. *In vitro* propagation of endangered plants could offer considerable benefits for the rapid cultivation of risk species, which have a limited reproductive capacity and exist in threatened habitats (Fay 1992).

Dictyospermum ovalifolium Wight. is a rare plant, endemic to the Hulical region of Western Ghats of India (Nayar and Sastry 1988). 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 were used in the treatment for allergies and skin diseases. The clearing of forest 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 of their mass propagation and conservation. An efficient tissue culture protocol for the mass multiplication was standardized in this species, through rapid axillary bud proliferation (Thoyajaksha and Rai 2001).

Tissue culture induced variations can be determined at the morphological, cytological, biochemical and molecular levels with several techniques. Molecular markers suitable for generating DNA profiles have proved to be an effective tool in assessing the genetic stability of regenerated plants. Three widely used polymerase chain reaction (PCR)-based markers are random amplified polymorphic DNA (RAPD; Williams *et al*. 1990), amplified fragment length polymorphisms (AFLP; Vos *et al*. 1995) and simple sequence repeats (SSR) or micro-satellites (Zietkiewicz *et al*. 1994). The choice of a molecular marker technique depends on its simplicity and reproducibility. Inter simple sequence repeat (ISSR) markers were recently shown to be specific, reproducible and sensitive for detecting variations among individuals between and within species (Bornet and Branchard 2001). The ISSR markers have also been used successfully to assess genetic stability among micropropagated plants (Joshi and Dhawan 2007). Screening the tissue culture derived plants using molecular markers will assist in reintroducing true-to-type plants (Heinze and Schimidt 1995) and protecting their genetic integrity. In this paper, we describe *in vitro* multiplication of *D. ovalifolium*, evaluation of gene-tic integrity of the micropropagated plants by using ISSR technique and its establishment in the original habitat.

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Abbreviations: ISSR - inter simple sequence repeat; PCR - polymerase chain reaction.
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Actively growing shoots of *Dictyospermum ovalifolium* were collected from the wild population near Hulical Ghats of Karnataka. Surface sterilized nodal segments measuring 1.5 cm collected from healthy young branches were established on Murashige and Skoog (MS) medium fortified with 6-benzyladenine (BA; 3.0 mg dm^{-3}).

Primary shoots formed *in vitro* were sectioned into single node pieces after removing the leaves. The nodal segments containing axillary buds were cultured on MS medium with 3.0 mg dm^{-3} BA for further multiplication. After 30 d, 4 - 5 shoot segments from the *in vitro* grown plants were excised from the shoot clump and transferred to MS medium supplemented with auxins indole-3-acetic acid (IAA ; 1.0 mg dm^{-3}) and indole-3-butryic acid (IBA; 2.0 mg dm^{-3}) for root induction. After 4 weeks on root induction medium, the rooted plantlets were transplanted to plastic pots containing *Soilrite*, and kept in growth chamber (*Sanyo*, Osaka, Japan) under temperature of 25 ± 2 °C and irradiance of 50 μmol m^{-2} s^{-1} with 16-h photoperiod for 4 weeks.

The reintroduction program was carried out in two different regions of reserve forest area near Hulical and Etthankatte of Western Ghats region in three phases. In the first phase 1 000 micropropagated plants were successfully transplanted, followed by 1000 plantlets during the second phase and more than 500 plants in the third phase which were reintroduced in the same area consequently. The establishment rate and mortality rate were recorded subsequently for two years.

The 14 tagged regenerated plants from the above stage of hardening in the growth chamber were selected randomly for screening their genetic integrity. All these plants were derived from a single mother plant. Total DNA was extracted from fresh, young leaves of mother plant and field grown tissue culture plants using the modified CTAB method described by Doyle and Doyle (1990) and Gawel and Jarret (1991). Quality and quantity of DNA were inspected by both gel electrophoresis and spectrometric assays using UV-visible double beam spectrophotometer (*LABOMED*, Culver, USA).

A total of 17 primers were initially tested using the donor plant DNA (in two replicates) as template to screen for suitable primers. Each reaction were done in a total volume of 0.02 cm^3 containing, 0.002 cm^3 1× assay buffer [10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 50 mM KCl and 0.01 % gelatin], 0.2 μM primer, 200 μM dNTP, 1.5 unit of Taq DNA polymerase and 50 ng genomic DNA. DNA amplifications was performed in thermocycler *UNO II* (*Biometra*, Göttingen, Germany), programmed for 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, 2 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 depended on the primers used.

The ISSR-PCR amplification products were analyzed by electrophoresis in 2 % (m/v) agarose (*Amersham*, Uppsala, Sweden) with 1× TBE buffer (Tris-borate-EDTA buffer) stained with ethidium bromide (10 mg cm^{-3}). The amplified products were visualized and photographed under UV transilluminator and documented using *Bioprofile Image Analysis System* (Vilber Lourmat, France). In all cases, λ DNA / *Eco*R I-*Hind* III Double digest (*Genei,* Bangalore, India) was used as molecular size marker. All the reactions were repeated twice to check reproducibility.

Table 1. Total number of amplified fragments and number of polymorphic bands generated by PCR using selected ISSR primers.

Primer	Sequence	Number of amplified fragments	Number of polymorphic bands	Size range [bp]
UM18C1	$(CT)_8AC$	5	0	564-4268
UM18C2	$(CT)_8GC$	4	0	560-3530
UM14C3	$(CA)_6AC$	3	0	831-3530
UM14T4	$(CA)_6GT$	8	1	560-4500
UM14G5	$(CA)_6AG$	2	0	564-2100
UM14C6	$(CA)_6GC$	9	0	200-3000
UM14G7	$(GT)_6GG$	2	0	2027-3530
UM14C8	$(GA)_6CC$	4	1	200-2027
UM11C9	$(CAC)_3GC$	6	0	200-4262
UM11C10	$(GAG)_3GC$	1	0	200
UM11G11	$(CTC)_3GC$	2	0	700- 831
UM11C12	$(GTG)_3GC$	2	0	2027-3530
UM19T13	$(GA)_9T$	2	0	200- 500
UM16A14	$(GACA)_4$	1	0	500

Amplified DNA bands were scored with all the selected ISSR primers (Table 1), and only clear and reproducible bands in the size range of 200 bp to 2.1 kb were scored. The bands were transformed into a binary character matrix, "1" for presence and "0" for absence of band, at a particular position. Pairwise distance matrices were computed based on the Jaccard's coefficient of similarity (Sneath and Sokal 1973). Data analysis was performed using the *NTSYS-pc* version 2.02a computer program package (Rohlf 1997). Dendrograms were constructed with the unweighted pair group method with arithmetic averaging (UPGMA; Rohlf 1993).

Using, MS medium fortified with BA (3.0 mg dm^{-3}), about 58.3 % of the nodal explants showed multiple shoot formation in about 4 - 5 weeks. At the same time, repeated subculturing of nodal segments from *in vitro* grown shoots helped to achieve continuous production of callus free healthy shoots. After being transferred onto the root induction medium 91.7 % of shoots, regenerated roots. More than 95 % of the intact plantlets survived after being transferred into pots in growth chamber. Five months later, the plants were transplanted into the field where they grew normally. Thus within a time frame of 6 months, a populations of >1 650 regenerated plants of *D. ovalifolium*, originating from a single mother donor plant, was established in the field. Most of the plants showed better establishment in areas of Hulical forest

region with survival rate of 88 % when compared to the Etthanakatte region with survival rate of 40.8 %. Although many workers have suggested the role of *in vitro* propagation of rare and endangered plants as an effective means of conservation (Anand 2003), limited reports on reintroduction of micropropagated plants in wild are available (Seeni and Latha 2000).

In the present study, genomic DNA was isolated from the randomly selected 14 micropropagated plants of *D. ovalifolium*. Of the 17 ISSR primers screened with the donor plant DNA template (replicated), 14 were found to generate highly reproducible patterns with multiple discrete bands in *D. ovalifolium* and were thus used for the analysis of the 14 regenerated plants. Indeed, all these 14 primers were found to generate monomorphism identical in two independent amplifications that were performed for all samples. Detailed information regarding the selected ISSR primers, total number of amplified fragments scored, number of polymorphic bands for each primer, size range of the amplified bands was summarized in Table 1. Twelve of the 14 primers produced monomorphic pattern, confirming the genetic uniformity of the micropropagated material. The representative amplification patterns obtained by the primers (UM18C1 and UM14C2) are shown in the Fig. 1. While each of the rest 2 primers generated polymorphic bands at least in one of the 14 individuals relative to the donor plant. The 14 tested primers yielded a total of 51 bands and corresponded to an average of 3.64 bands per primer. Out of the 51 amplified bands scored, 2 were polymorphic among the 14 individual plants and the bands ranged from 200 bp to 2.1 kb in size. The polymorphisms included the presence of a novel band in primer UM14T4 in regenerants plant no. 3 and 4 at approximate size of 700 bp and the absence of 200 bp size fragment in primer UM14C8 in regenerants plant no. 1, 2, 5, 8 and 9.

The ISSR data were used to calculate genetic similarity among the 14 individuals in a pair wise manner. The similarity coefficient of dendrogram among the plants ranged from 0.882 to 1.000 with a mean of 0.978. Associations among the plants (a cluster analysis) were depicted based on the Jaccard's coefficient between 15 individuals (14 regenerated and 1 donor plant) by means of UPGMA, which clustered the plants into

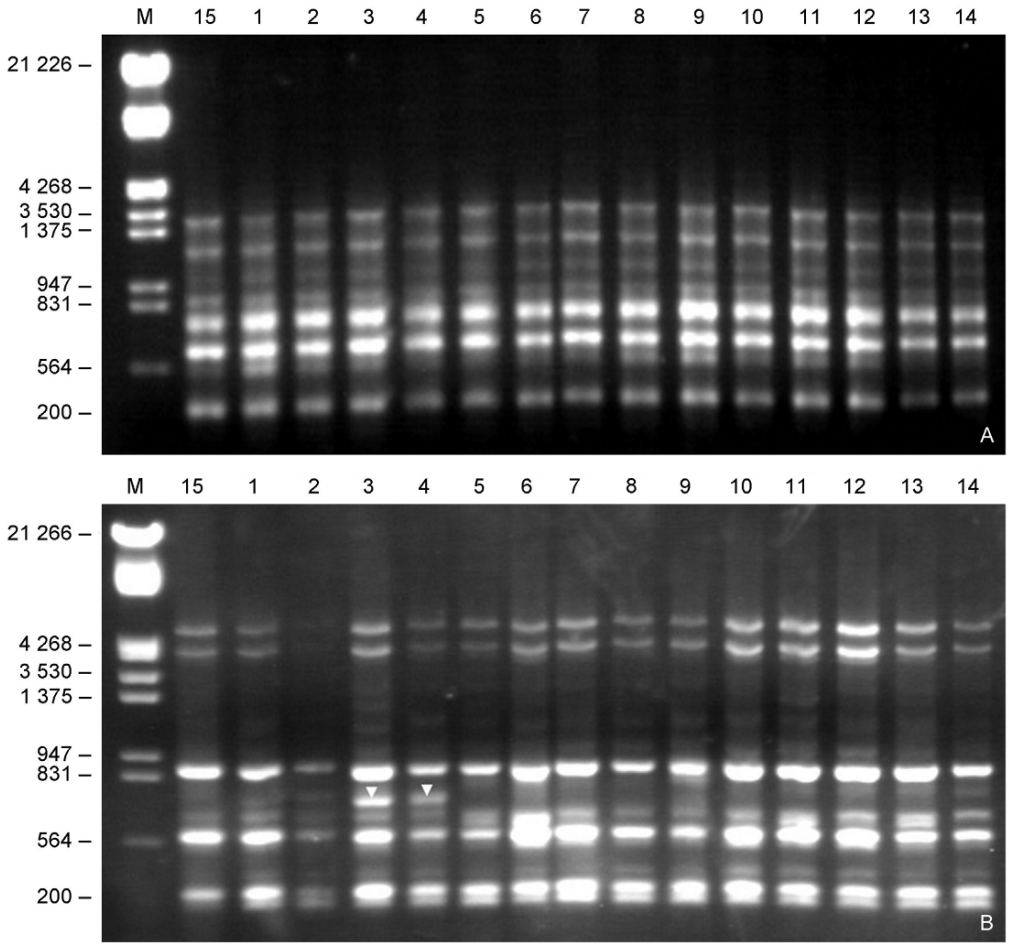

Fig. 1. *A* - ISSR products generated from fourteen *in vitro* plants and mother plants of *D. ovalifolium* amplified with UM18C1. *Lanes*: M - molecular size marker, 15 - donor plant, 1 - 14 plant regenerated from the axillary buds. *B* - Examples of polymorphism of regenerants 3 and 4. Polymorphic DNA fragments are identified in UM18C2 by an *arrow.*

four groups with the donor plant in the second group. The 97 % of the plants showed similarity and were clustered into two distinct groups, except two plants Do-13 and Do-8 which showed some variation.

The overall polymorphism frequency detected in *D. ovalifolium* was at 3.92 %. This polymorphism is relatively low and the polymorphic bands in the regenerants included either loss of original band present in the donor plant or gain of novel band. This clearly indicated that complete genetic fidelity is lacking among the regenerants. The small genetic variation in DNA observed by ISSR analysis may be due to various factors, such as the *in vitro* process and its duration, auxins-to-cytokinin concentration and their ratio (hormonal balance), other nutritional conditions and *in vitro* stress, all of which are known to induce somoclonal variation (Devarumath *et al.* 2002). Similar findings on genomic variations in phenotypically normal plant regenerants have been made previously in other plant species (Rahman and Rajora 2001, Carvalho *et al*. 2004, Guo *et al*. 2006 a,b, Feyissa *et al*. 2007)

ISSR has been proven as more useful molecular marker for genotyping and for studying genetic diversity in plants compared with widely used RAPD marker (Guo *et al.* 2006b). ISSR has several advantages, particularly in reproducibility and higher informative nature (Nagaoka and Ogihara 1997). ISSR has been successfully used to detect variations in several plants including *Coffea arabica* (Rani *et al.* 2000), tea (Devarumuth *et al*. 2002), banana (Ray *et al*. 2006), *Codonopsis lanceolate* (Guo *et al*. 2006a) and *Robinia ambigura* (Guo *et al*. 2006b). Our study supports the earlier reports that plants derived from organized meristem collected may not always be gene-tically true-to-type. The ISSR analysis of *D. ovalifolium* revealed a low variation among rege-nerants, similar to *Gypsophila paniculate in vitro* regenerated plants (Rady 2007).

In conclusion, the techniques described here for the micropropagation of *D. ovalifolium* through axillary bud multiplication facilitates the rapid propagation of this rare plant species. The results demonstrate that ISSR markers can be applied to evaluate the genetic stability of regenerants for the conservation of genetic resources of Western Ghats of India.

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