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Genetic Stability in Micro-clones of *Celastrus paniculatus* Willd. Derived from Different Pathways of Micropropagation as Revealed by RAPD Profiles

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ABSTRACT

Micro-clones of *Celastrus paniculatus* Willd. derived from two different micropropagation pathways (axillary branching and adventitious regeneration) were subjected to molecular screening using random amplified polymorphic DNA (RAPD) markers. Of 20 decamer primers used, amplification products were obtained with 11 primers only. The number of bands varied from 5 to 12. Irrespective of the micropropagation pathway, no variability could be detected in the regenerants suggesting that both pathways are reliable and could be used for large-scale production of this plant.

Keywords: adventitious regeneration, axillary branching, genetic fidelity, molecular markers, variability
Abbreviations: PCR, polymerase chain reactions; **RAPD**, random amplified polymorphic DNA

INTRODUCTION

Micropropagation has been widely applied for large-scale production of plantlets *in vitro* (Pierik 1987). The sustainability of micropropagation, especially for commercial cultivation, depends upon the production of true-to-type plantlets and maintenance of genetic integrity of the propagules. Micropropagation through axillary branching is generally considered to be immune to genetic changes (Shenoy and Vasil 1992) whereas *in vitro* regeneration through adventitious budding may cause genetic variability (Phillips *et al*. 1994). Molecular markers such as restriction fragment length polymorphism (RFLP) (Botstein *et al*. 1980), random amplified polymorphic DNA (RAPD) (Williams *et al*. 1990), amplified fragment length polymorphism (AFLP) (Vos *et al*. 1995), simple sequence repeats (SSR) (Tautz *et al*. 1989), inter-simple sequence repeats (ISSR) (Zietkiewicz *et al*. 1994) and a few others have been widely used for the screening of tissue culture raised plantlets (Chowdhury *et al*. 1994; Rani and Raina 1998; Martins *et al*. 2004; Modgil *et al*. 2005).

Celastrus paniculatus Willd. (Family: Celastraceae), commonly known as 'Malkangni' or 'Jyotishmati', is a large woody climbing shrub valued for its immense medicinal properties contained in its various parts (Thakur *et al*. 1989). The chief phytoconstituents of medicinal value reported in *C. paniculatus* include malkangunin (a sesquiterpene polyester), celapanin, celapanigin and celapagin (sesquiterpene alkaloids) and celastrol, pristimerin, zeylasterone and zeylastral (quinine–methide and phenolic triterpenoids) (Anonymous 2002). In *C. paniculatus in vitro* shoot proliferation and plantlet regeneration have been reported using different pathways and a variety of explant sources (Bilochi 2001; Nair and Seeni 2001; Arya *et al*. 2002; Sharda *et al*. 2003; Rao and Purohit 2006). The present investigation was aimed to assess genetic stability/variability in micro-clones of *C. paniculatus* regenerated through different pathways of micropropagation using different explant sources.

MATERIALS AND METHODS

Plant materials

Explants of *C. paniculatus* were collected from a nursery grown 4 year old plant. Nodal explants (ca. 3.0-3.5 cm) were used to establish shoot cultures through enhanced axillary branching as described by Bilochi (2001). *In vitro* multiplied shoots on standard multiplication medium provided internodes and leaves to obtain *in vitro* shoot bud differentiation as described earlier (Rao and Purohit 2006; Rao 2007) (**Fig. 1**). All the cultures were maintained under the same incubation conditions and multiplied by repeated subculturing at a fixed frequency of 21 days. After ten subcultures, micropropagules were randomly selected for RAPD analysis. Total genomic DNA was extracted from leaf material of field grown mother plant and *in vitro* derived plantlets regenerated from different explant sources such as nodal segment, culture derived internodes and leaves.

Extraction and quantification of genomic DNA

Total genomic DNA from leaf samples was extracted using modified cetyle trimethyl ammonium bromide (CTAB) procedure (Lodhi *et al*. 1994). The impurities of RNA were removed by the treatment of RNase A. All the DNA samples were digested with 100 µg/ml RNase A for 30 min at 37°C, extracted once with chloroform: isoamyl alcohol (24:1), precipitated by adding 0.1 vol of sodium acetate (3 M) and 2.5 vol of chilled ethanol, washed with 70% ethanol and resuspended in 100 µl 1 x TE. The quality of genomic DNA was checked by agarose gel electrophoresis on 0.8% agarose gel (w/v), stained with 0.5 µg/ml ethidium bromide. DNA concentration was estimated spectrrophotometrically (UV-Vis Spectrophotometer, Pharmaspec UV-1700, Shimadzu, Japan) by measuring the absorbance at 260 nm. The original DNA samples were then diluted to 5.0 ng/µl for PCR.

Amplification conditions and primer selection

RAPD profiles were performed as described by Williams *et al*. (1990) with minor modifications. PCR reactions were carried out

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Fig. 1 (**A**) Mature field grown plant of *Celastrus paniculatus*. (**B**) Axillary shoot bud proliferation from mature node explants. (**C**) Adventitious shoot bud differentiation from culture derived internod explant. (**D**) Adventitious shoot bud differentiation from culture derived leaf explant. (**E**) Profusely multiplying shoot cultures.

in 0.2 ml polypropylene PCR tubes (Bangalore Genei, India) using Thermal Cycler (Master Cycler Personal, Eppendorf). Each 20 µl reaction mixture contained 1 x Taq buffer (100 mM Tris-cl (pH 9.0), 500 mM KCl, 15 mM $MgCl_2$ and 0.1% gelatin), 2.5 mM $MgCl_2$, 0.2 mM dNTPs (Bangalore Genei, India), 20 pmol oligonucleitide primers (Sigma Genosys, India), 1U Taq DNA polymerase (Bangalore Genei, India) and 25 ng template DNA. The reactions were subjected to initial denaturation at 94°C for 4 min followed by 30 amplification cycles, each consisting of 1 min at 94°C (denaturation step), 1 min at 37°C (annealing step) and 2 min at 72°C (extension step) with a final extension of 7 min at 72°C. The amplification products were separated on 1.5% agarose (w/v) gel, stained with 0.5 µg/ml ethidium bromide. DNA ladder 1 Kb (Bangalore Genei, India) and 100 bp DNA ladder (Bangalore Genei, India) were mixed and used as molecular weight marker for comparison of amplified products. Gels were photographed under UV light using a Gel Documentation System (DP 001. FDC, Consort). All reactions were repeated thrice to confirm the results.

Twenty arbitrary decamer oligonucleotide primers (RUF201-RUF 220; Sigma Genosys, India) were used for screening and only those primers were selected for the present study, which provided satisfactory and reproducible amplification products under similar conditions.

Data analysis

All reactions were performed thrice and only consistently reproducible and well resolved bands were considered for the analysis. Amplified fragments were scored as '1' or '0' for presence or absence of bands on the gel. DNA polymorphism was calculated and given as percentage of the total number of bands produced in RAPD profiles.

RESULTS AND DISCUSSION

In the present investigation RAPD profiles of *C. paniculatus* were obtained employing twenty random decamer primers. Out of twenty primers screened, eleven produced clear and reproducible amplification products (**Table 1**). No variation was observed in any of the profiles (**Fig. 2**). The selected primers yielded a total of 97 amplified fragments with an average of 8.81 bands per primer. Number of bands varied from 05 (RUF216) to 12 (RUF210). The size of the bands produced by these primers ranged from 190 bp (RUF208) to 2000 bp (RUF217). RAPD profiles indicated uniformity among all the micropropagules of *C. paniculatus* irrespective of the explant source and pathway of micropropagation as observed in the form of monomorphic ban-

Table 1 RAPD analysis of genomic DNA extracted from mother plant and micropropagated plants (regenerated through different explant sources) of *C. paniculatus* using eleven random decamer primers.

Primers	Sequence 5′-3′	Total № amplified fragments	Fragment size range (bp)
RUF202	TTGGCGGCCT	9	400-1900
RUF205	TGGGTCCCTC	8	350-1800
RUF206	ACGGGCCAGT	8	350-1700
RUF207	CAGGCCCTTC	11	370-1500
RUF208	GTGACCTAGG	8	190-900
RUF210	TGCCGAGCTG	12	350-1500
RUF211	GGGTAACGCC	11	200-1000
RUF213	TTCGGGCCGT	11	550-1900
RUF215	GCTGCGTGAC	7	250-750
RUF216	CAGCGAACTA	5	400-1500
RUF217	CGACTCACAG	8	450-2000
		Total 97	

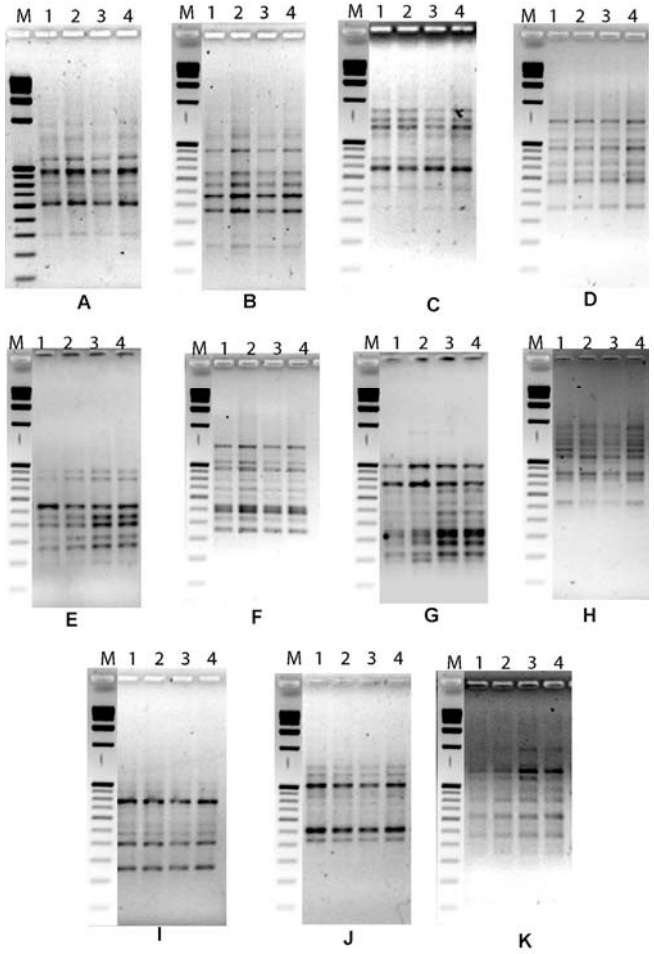

Fig. 2 RAPD profiles generated from DNA isolated from *Celastrus paniculatus* plants obtained from different micropropagation pathways using three explant sources. Lanes: 1, mother plant; 2, mature node; 3, culture-derived internode; 4, culture-derived leaves. Select primers used:**A**, RUF202; **B**, RUF205; **C**, RUF206; **D**, RUF207; **E**, RUF208; **F**, RUF210; **G**, RUF211; **H**, RUF213; **I**, RUF215; **J**, RUF216; **K**, RUF217); M, molecular weight marker (1 kb ladder; Bangalore Genei, India).

ding patterns and were similar to the mother plant.

Although a great degree of polymorphism has been reported in natural populations of *C. paniculatus* (Raju and Prasad 2007) based on RAPD markers, however the absence of polymorphism in the present study corroborated RAPD analysis in micropropagated plants of enhanced axillary branching derived *Camellia* species (Devarumath *et al*. 2002), *Musa acuminata* (Lakshmanan *et al*. 2007) and *Foeniculum vulgare* (Bennici *et al*. 2004) regenerated through adventitious mode of propagation. It is well established that axillary bud development is not expected to generate genetic variability, since it uses the normal ontogenetic route for branch growth through lateral meristem (Zuchhi *et al*. 2002) and are more resistant to genetic changes that might occur during cell division or differentiation under *in vitro* conditions (Shenoy and Vasil 1992). In the present case the absence of variability among regenerants of adventitious bud differentiation may be explained on the basis that the explants were taken from a single source as has been reported in case of almond (Martins *et al*. 2004). Carvalho *et al*. (2004) used RAPD fingerprints to study genetic stability of *in vitro* propagated plants of chestnut hybrids regenerated through axillary branching and no polymorphism was detected between *in vitro* plants and donor plants. Similarly, complete genetic stability was confirmed in *Arachis retusa* clones obtained from cotyledons and embryo axes (Gagliardi *et al*. 2007). Feyissa *et al*. (2007) used RAPD markers to assess genetic stability in micropropagated *Hagenia abyssinica* plants of axillary and adventitious origin and showed identical banding patterns except two unique bands out of total 115 bands. Similar results with very low genetic variation have also been reported in axillary branching derived *Tectona grandis* (Gangopadhyay *et al*. 2003), leaf explants derived *Drocera anglica* (Kawiak and Lojkowska 2004) and beet plantlets (Munthali *et al*. 1996).

In contrast, somaclonal variation has been reported in a number of cases such as micropropagated plants of *Populus tremuloides* (Rahman and Rajora 2001), *Syzygium travancoricum* (Anand 2003), *Curcuma amada* (Prakash *et al*. 2004), *Gypsophila paniculata* (Rady 2006) and *Actinidia deliciosa* (Pardo *et al*. 2007) using molecular markers. The reasons for these apparent results showing somaclonal variations can be related to different factors such as explant sources, ploidy level, culture methods and environment and *in vitro* culture age (Rani and Raina 2000).

It can be concluded that in *C. paniculatus* both the micropropagation pathways using different explant sources are equally reliable to produce a large number of genetically uniform plantlets. The results indicated that the recovered shoots were genetically stable at the assessed genomic regions.

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