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Genetic Stability Studies in Micropropagated Date Palm (*Phoenix dactylifera* L.) Plants using Microsatellite Marker

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ABSTRACT : Sixteen microsatellite markers (simple sequence repeat (SSR) markers) were employed to examine the genetic stability of 27 randomly chosen date palm (*Phoenix dactylifera* L.) plants produced through somatic embryogenesis with upto forty two *in vitro* subcultures. No microsatellite DNA variation was observed among all micropropagated plants. Our results indicate that the micropropagation protocol used for rapid *in vitro* multiplication is appropriate and suitable for clonal propagation of date palm and corroborated that somatic embryogenesis can also be used as one of the safe modes for production of true-to-type plants of date palm. This is the first report on the use of microsatellite DNA markers to establish the genetic stability in micropropagated date palm plants.

Keywords : *Phoenix dactylifera* L., Genetic stability, SSR, microsatellite

INTRODUCTION

Date palm *(Phoenix dactylifera* L.) (2n=2x=36) is a dioecious, perennial, monocotyledon fruit tree that belongs to family Arecaceae. Date palm is an important fruit tree of arid and semi-arid regions of the world due to its high tolerance to environmental stresses. This fruit tree also has the potential of generating good income and foreign exchange from unproductive tracts of land (Hassan et al., 2006). Though it is propagated traditionally through offshoots or suckers, this propagation method has the several limitations like- offshoots are produced in a limited number for a certain period in the lifetime of a young palm tree and transmission of disease-causing pathogens and insects along with the offshoot (Taha et al., 2003; Sudhersan and Aboel Nil, 2004). Thus, the traditional method of vegetative propagation through offshoot being slow, laborious, time-consuming and expensive (Sudhersan and Aboel Nil, 2004) led to focus on development of micropropagation techniques for rapid clonal production of true-to-type plants through *in vitro* techniques. Propagation of date palm through *in vitro* techniques has been reported by several researchers using different explants sources as well as regeneration pathways (Tisserat, 1979; Sharma et al.,1984; Dass et al.,1989; Bekheet et al., 2001; Eke et al., 2005) However, scaling up of any micropropagation protocol is severely hindered due to incidences of somaclonal variations, *e.g.* as in case of oil palm, where aberrant flowering patterns were observed among the regenerated plants (Matthes et al., 2001). Somaclonal variations can occur for various morphological, physiological, disease resistance and other traits as well as for biochemical and molecular genetic markers (Phillips et al., 1994; Rani and Raina, 2000). As gross morphological variations are expected to occur at a much lower frequency than cryptic (e.g., DNA level) variations (Evans et al., 1984), the absence of visible variation does not preclude the absence of all variation among the micropropagated progeny. Hence, a stringent quality check in terms of genetic similarity of the tissue culture raised plants becomes mandatory. Identification of off-types and genetically true-to-type of mother plant at an early stage of development is considered to be very useful for quality control in plant tissue culture.

Biochemical markers may be used for examining cryp-

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tic somaclonal variation, but these markers are limited in number and levels of polymorphism both, further these markers are developmentally regulated and affected by external environment in their expression. DNA markers are a more suitable means for examining the clonal fidelity as they are more informative and not developmentally regulated or affected by the external environment. Among the DNA markers, random amplified polymorphic DNA (RAPD) technique has limited reproducibility (Riedy et al., 1992; Ellsworth et al., 1993) while amplified fragment length polymorphism (AFLP) is quite cumbersome and not amenable to high-throughput screening. Moreover, RAPD and AFLP are dominant diallelic markers, thus, individual parental alleles cannot usually be differentiated by these markers in diploid organisms. Therefore, these markers are not quite informative enough for examining somaclonal variation. Highly polymorphic and reproducible DNA markers are the most suitable markers for detecting somaclonal variation. The sensitivity, reproducibility, co-dominance and strong discriminatory power of microsatellite DNA/SSR (simple sequence repeat) markers (Rajora et al., 2001) make them particularly suitable for detecting somaclonal variation, but their application in the study of somaclonal variation has been rather quite limited (Chowdari et al., 1998). SSR marker has been successfully applied to detect the genetic similarities or dissimilarities in micropropagated material in various other plant species (Rahman and Rajora, 2001). The objective of the present study was to monitor the genetic stability of long term (168 weeks) micropropagated plants of date palm using the SSR markers. To our knowledge, this is the first assessment of clonal fidelity in long term micropropagated plants of date palm species using microsatellite DNA marker.

Materials and Methods

Plant material

A protocol for micropropagation through somatic embryogenesis was employed for a large-scale commercial production of this fruit bearing tree (unpublished). Growing shoot tip and primordial leaf explants from the offshoots were used as explants and aseptic cultures were established by treating these explants with 0.1% $HgCl_2$ for 12 min followed by rinsing with sterilized distilled water (three times). The shoot tip and primordial leaf cultures were then subjected to callus induction on MS medium (Murashige and Skoog, 1962) supplemented with 33.75 μM 6-benzyl aminopurine (BAP), 42.50 μM indole-3-acetic acid (IAA), 2% sucrose, and 0.5% charcoal (callus induction medium). The callus thus obtained was subjected to MS medium containing 100 μM 2, 4-dichlorophenoxy acetic acid (2, 4-D), 2% sucrose and 0.5% charcoal for induction of somatic embryogenesis (somatic embryo induction medium). The mature somatic embryos were then subjected to germination and plantlets formation on MS medium containing 11.2 μM IAA, 25 μM indole-3-butyric acid (IBA), 2% sucrose and 0.5% charcoal (germination medium). A hardening success of 94% was obtained. Twenty seven plants were randomly chosen, for the DNA based genetic stability analysis, from over 2000 micropropagated plants produced after total forty two subcultures on different media.

DNA extraction

Total DNA was extracted separately from leaf tissues of 27 randomly chosen micropropagated plants and a mother plant (control), using the method described by Doyle and Doyle (1990). 0.1 g of leaf tissue was ground in liquid nitrogen and taken in to a 2 mL microcentrifuge tube. To the ground sample 0.5 mL of extraction buffer (2% CTAB, 100 mM Tris-HCl, 3.5 M NaCl, 20 mM EDTA, 0.2 M β-Mercaptoethanol, 2% PVP, pH 8.0.) was added and incubated at 65℃ for 90 min. The above sample was extracted with equal volume of chloroform: isoamyl alcohol (24:1) and supernatant was transferred in to a new tube. The sample was treated with RNase and extracted with Tris saturated phenol. The supernatant after extraction with Tris saturated phenol was taken and extracted further with chloroform:isoamyl alcohol (24:1) twice, and precipitated with 80% of ethanol. The pellet

Table 1. List of SSR primers used in the detection of genetic stability in micropropagated plants of date palm.

Sr. No.	Primer name	Motif repeat	Annealing Temperature (℃)	Primer sequence (5'-3')	
				Forward	Reverse
1	mPdCIR010	(GA)22	60	ACCCCGGACGTGAGGTG	CGTCGATCTCCTCCTTTGTCTC
2	mPdCIR015	(GA)15	60	AGCTGGCTCCTCCCTTCTTA	GCTCGGTTGGACTTGTTCT
3	mPdCIR016	(GA)14	52	AGCGGGAAATGAAAAGGTAT	ATGAAAACGTGCCAAATGTC
4	mPdCIR025	(GA)22	57	GCACGAGAAGGCTTATAGT	CCCCTCATTAGGATTCTAC
5	mPdCIR032	(GA)19	52	CAAATCTTTGCCGTGAG	GGTGTGGAGTAATCATGTAGTAG
6	mPdCIR035	(GA)15	59	ACAAACGGCGATGGGATTAC	CCGCAGCTCACCTCTTCTAT
7	mPdCIR044	(GA)19	52	ATGCGGACTACACTATTCTAC	GGTGATTGACTTTCTTTGAG
8	mPdCIR048	(GA)32	54	CGAGACCTACCTTCAACAAA	CCACCAACCAAATCAAACAC
9	mPdCIR050	(GA)21	53	CTGCCATTTCTTCTGAC	CACCATGCACAAAAATG
10	mPdCIR057	(GA)20	59	AAGCAGCAGCCCTTCCGTAG	GTTCTCACTCGCCCAAAAATAC
11	mPdCIR063	(GA)17	52	CTTTTATGTGGTCTGAGAGA	TCTCTGATCTTGGGTTCTGT
12	mPdCIR070	(GA)17	60	CAAGACCCAAGGCTAAC	GGAGGTGGCTTTGTAGTAT
13	mPdCIR078	(GA)13	56	TGGATTTCCATTGTGAG	CCCGAAGAGACGCTATT
14	mPdCIR085	(GA)29	52	GAGAGAGGGTGGTGTTATT	TTCATCCAGAACCACAGTA
15	mPdCIR090	(GA)26	60	GCAGTCAGTCCCTCATA	TGCTTGTAGCCCTTCAG
16	mPdCIR093	(GA)16	55	CCATTTATCATTCCCTCTCTTG	CTTGGTAGCTGCGTTTCTTG

was air dried and dissolved in 100 μL of TE buffer. Purified total DNA was quantified and its quality was verified by spectrophotometer and each sample was diluted to 20 ng/μl with TE buffer and stored at 4℃.

PCR conditions for microsatellites (SSR) amplification

A set of 16 date palm specific SSR primer pairs developed by Billote et al. (2004) (Table 1) were used in the study. Each SSR amplification was performed in a total volume of 25 μl containing 5 μl DNA (100 ng), 12.5 ml master mix (Genei, Bangalore, India), 1 μl of 10 pmol of SSR primer pairs each (Operon Technologies, Alameda, California). The amplification reaction consisted of an initial denaturation step at 94℃ for 5 min, followed by 35 cycles of 1 min at 94℃ (denaturation), 1 min at a specific annealing temperatures (Table 1) and 1 min at 72℃ (extension) followed by a final extension step at 72℃ for 5 min. Amplifications were performed in a thermocycler (Biometra, Germany). DNA amplicons were separated on 2% agarose gel using 1x TBE buffer and stained with ethidium bromide. Gels were visualized using a gel documentation system (Bio-Rad, Hercules, California). The size of the amplicons was estimated by comparing with 100 bp DNA ladder (Fermentas, Germany).

Results and discussion

A method of micropropagation through somatic embryogenesis in date palm was developed using shoot tip as well as primordial leaves as explants. The explants were incubated on a callus induction medium for a period of seven months, during which the cultures were subcultured on fresh medium with same composition at an interval of four weeks for induction and multiplication of embryogenic callus (Fig 1a). The resulting embryogenic callus was subjected to a somatic embryo induction medium for a period of four months, with subcultures on fresh medium with same composition at an interval of four weeks for the formation as well as maturation of somatic embryos (Fig. 1b). The resulting matured somatic embryos were germinated on a germination medium for plant formation

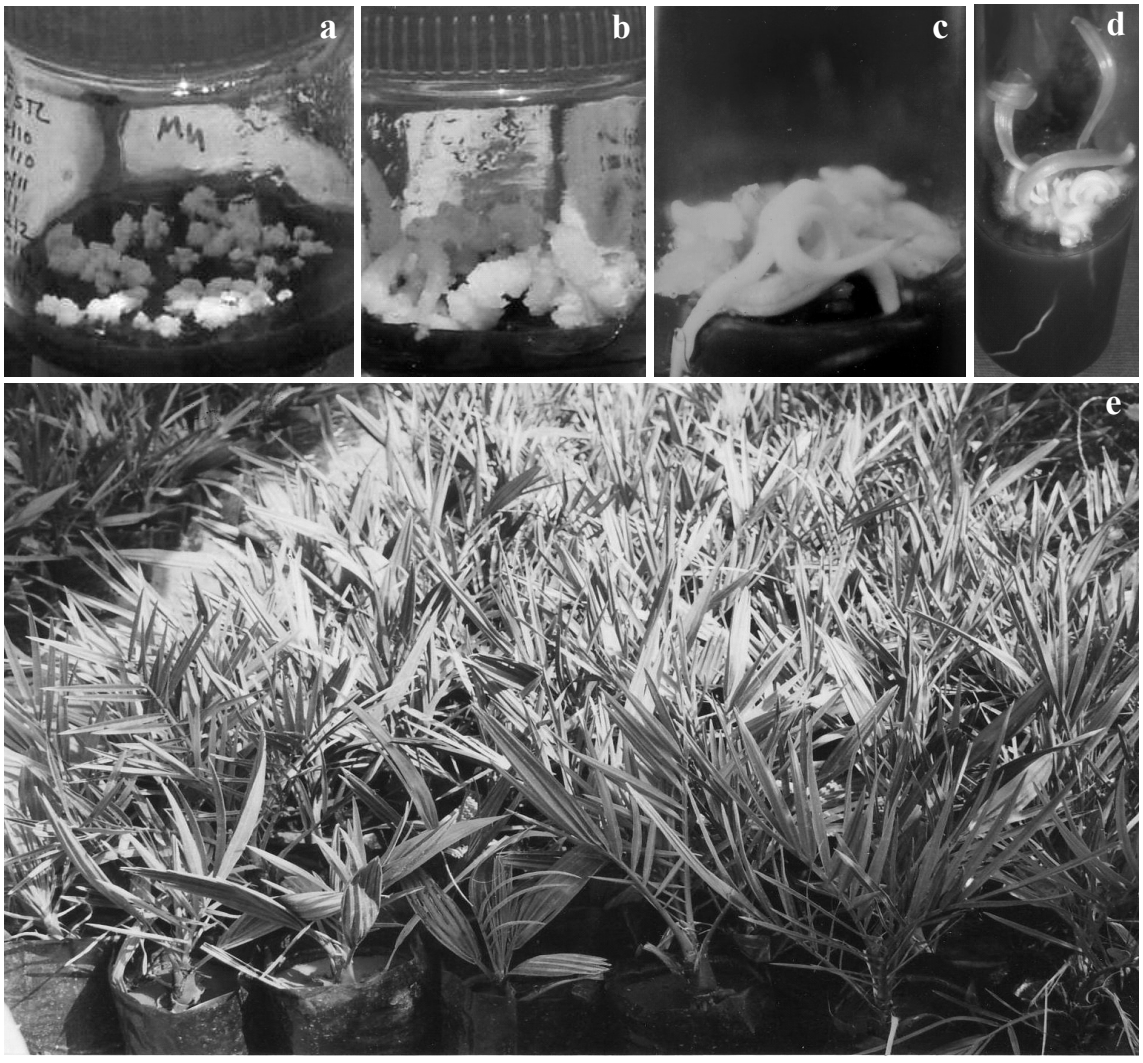

Fig. 1. Micropropagation of date palm through somatic embryogenesis. (a) Induction of callus. (b) Development of embryogenic callus. (c) Germination of somatic embryo. (d) Regenerated plantlets. (e) Hardened plants in nursery.

(Fig. 1c and d). Similar procedures have been reported by other researchers (Sharma et al., 1984; Dass et al., 1989; Eke et al., 2005).

In order to test the genetic fidelity of micropropagated date palm plants (Fig. 1e) sixteen microsatellite DNA marker (SSR) primers pairs were used in 27 randomly chosen plants. However, no microsatellite DNA variation was observed in any of the micropropagated plants studied (Fig. 2). An example of the monomorphic amplicons obtained for SSR marker is shown (Fig. 2a, b). Thus, these results confirm that somatic embryogenesis for micropropagation of date palm maintains the genetic fidelity even after prolonged period of 168 weeks (three years) under *in vitro* conditions.

The results of our study corroborate the fact that somatic embryogenesis is one of the safest modes of micropropagation to produce true-to-type plants. There are many reports in literature, which cite similar results (Andrea et al., 2004; Lopes et al., 2006). Somatic embryogenesis is usually considered to be one of the best multiplication methods as there is stringent genetic control throughout the somatic embryo formation and selection pressure against the abnormal types is considerably high (Leroy et al., 2000). Off-types have also been detected from micropropagated plants in *Pinus thunbergii* (Goto et al., 1998), pecan somatic embryos (Vendrame et al., 1999),

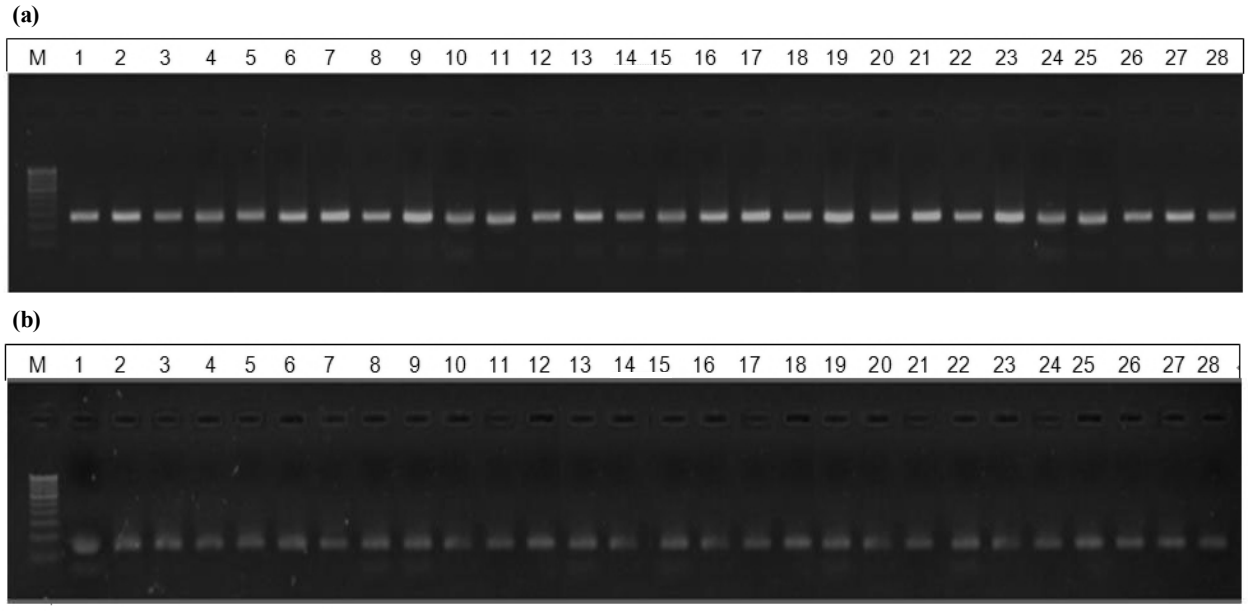

Fig. 2. SSR amplification products obtained with (a) primer No. 10 and (b) primer No. 13. *Lane M*, represents 100 bp ladder; *lane 1*, represents mother plant; *lanes 2-28*, represent tissue cultured raised plants at the 42nd cycle of transfer.

Populus tremuloides (Rahman and Rajora, 2001) and tea clones (Devarumath et al., 2002). Hence, it becomes imperative to check the genetic fidelity of the micropropagated plants in order to produce clonally uniform plants while using different techniques of micropropagation.

Variations induced in tissue cultured plants is likely to be reflected in the banding profiles developed by employing different marker systems. However, the reliability and efficiency of molecular markers in detecting large-scale genome rearrangements have been frequently questioned. However, the use of microsatellite DNA / SSR markers, in particular, is considered suitable for detecting somaclonal variation because of their abundance, sensitivity, reproducibility, co-dominance and strong discriminatory power. So, in the present study, we had adopted microsatellite DNA markers, for the identification of somaclonal variation in micropropagated date palm plants. Since no variations were observed in the banding patterns in micropropagated plants as compared to the mother plant, we conclude that, our micropropagation protocol, through indirect somatic embryogenesis, can be effectively used for a considerable length of time under *in vitro* conditions (168 weeks) without much risk of inducing genetic instability / somaclonal variations in date palm. The method also offers great promise for mass propagation of true-to-type date palm plants from shoot tip and primordial leaf.

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