Assessment of genetic fidelity of micropropagated date palm (*Phoenix dactylifera* L.) plants by RAPD and ISSR markers assay

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ABSTRACT

RAPD (Random Amplified Polymorphic DNA) and ISSR (Inter-Simple Sequence Repeats) markers assay were employed to validate the genetic stability of date palm (*Phoenix dactylifera* L.) plants multiplied through somatic embryogenesis with upto forty two *in vitro* subcultures. Out of the 160 RAPD and 21 ISSR primers screened, 30 RAPD and 12 ISSR primers produced a total of 347 (246 RAPDs + 101 ISSRs) clear, distinct and reproducible amplicons, which were monomorphic across all micropropagated plants (27) studied. Thus, a total 8592 bands (number of plants analysed x number of amplicons with all the primers) were generated which exhibited homogeneous banding patterns with both RAPD and ISSR markers. These results indicate that the micropropagation protocol developed by us for rapid *in vitro* multiplication is appropriate and suitable for clonal propagation of date palm and corroborated the fact that somatic embryogenesis can also be used as one of the safest modes for production of true-to-type plants. [Physiol. Mol. Biol. Plants 2010; 16(2) : 207-213] E-mail : nitishbt1@rediffmail.com

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INTRODUCTION

Date palm (*Phoenix dactylifera* L. Arecaceae) ($2n=2x=36$) is a dioecious, perennial, monocotyledonary fruit bearing tree, with a very slow growth rate and a late reproductive phase. Palm trees are an excellent candidate for cultivation in harsh climatic conditions of arid and semi-arid regions of the world due to their high tolerance to environmental stresses where no other crops give economic returns. This fruit crop also has the potential of generating good income and foreign exchange from unproductive tracts (Hassan *et al.*, 2006). Date palms are commonly propagated by offshoots as this maintains the genetic integrity of the cultivars but offshoots are produced in limited numbers during a date palm's life span (Taha *et al.*, 2003). Therefore, date palm improvement programme by modern methods of agro-biotechnology are of interest worldwide. This has increased the importance of developing *in vitro* methods

to facilitate large scale production of true to type plants. 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 occur mostly in response to the stresses imposed on the plant under *in vitro* conditions and is manifested in the form of DNA methylations, chromosome rearrangements and point mutations (Phillips *et al.*, 1994). 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. Therefore, it is necessary to check the genetic uniformity of micropropagated plants by molecular techniques.

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Analyses of isozymes patterns of specific enzymes provide a convenient method for detection of genetic changes but are subjected to ontogenic variations. Such methods have limited applications due to their limited numbers and only those DNA regions coding for proteins can be sampled. Tissue and environment independence of DNA based markers have made them more reliable over morphological and isozyme markers. Among the various DNA-based markers, amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism (RFLP) can be used for screening of genetic stability/unstability in tissue culture raised plants, however, these methods involve the use of expensive enzymes, radioactive labeling and extensive care and hence, appear unsuitable. Random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) markers on the other hand, require only small amounts of DNA sample without involving radioactive labels and are simpler as well as faster. RAPD has proven to be quite efficient in detecting genetic variations, even in closely related organisms such as two near isogenic lines (NIL). ISSR technique is also a very simple, fast, cost-effective, highly discriminative and reliable (Reddy *et al.*, 2002). RAPD and ISSR markers have been successfully applied to detect the genetic similarities or dissimilarities in micropropagated material in various plants (Venkatachalam *et al.*, 2007; Joshi and Dhawan, 2007; Santos *et al.*, 2008; Ahmed *et al.*, 2009; Huang *et al.*, 2009). The objective of the present study was to monitor the genetic stability of long term (168 weeks) micropropagated plants of date palm using the RAPD and ISSR markers.

MATERIALS AND METHODS

Plant material

Micropropagation protocol by somatic embryogenesis was standardized for date palm and employed for a large-scale commercial production of this fruit bearing tree (unpublished). Growing shoot tip and primordial leaf explants from suckers 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 2 % sucrose, 33.75 μM 6-benzyl aminopurine (BAP), 42.50 μM indole-3-acetic acid (IAA) and 0.5 % charcoal (callus induction medium). The induced callus was subjected to MS

medium containing 100 μM 2, 4-dichlorophenoxy acetic acid (2, 4-D) and 0.5 % charcoal for induction of somatic embryogenesis (somatic embryo induction medium). The mature somatic embryos were subjected to germination and plantlets formation on MS medium containing 11.2 μM IAA, 25 μM indole-3-butyric acid (IBA) and 0.5 % charcoal (germination medium). A hardening success rate of 94 % was obtained. Twenty seven plants were randomly chosen, for the DNA based genetic stability analysis, from over 300 micropropagated plants produced after forty two subcultures.

DNA extraction and PCR amplification conditions

Total DNA was extracted from leaf material of 27 micropropagated plants and a mother plant (control), following the method described by Doyle and Doyle (1990). 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 °C.

RAPD amplification was performed in a volume of 25 μl containing 5 μl DNA (100 ng), 12.5 ml master mix (Genei, Bangalore, India), 1 μl of 10 pmol of 10-mer oligodeoxynucleotide primer (Operon Technologies, Alameda, California) (Table 1). The amplification reaction consisted of an initial denaturation step at 94 °C for 5 min, 35 cycles comprising denaturation at 94 °C for 1 min, annealing at 38 °C for 1 min and extension at 72 °C for 1 min and a final extension step at 72 °C for 5 min.

ISSR amplifications were performed in a volume of 25 μl containing 5 μl DNA (100 ng), 12.5 ml master mix (Genei, Bangalore, India), 1 μl of 10 pmol of ISSR primer (UBC Primers, MWG Biotech, Germany) (Table 2). The amplification reaction consisted of an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of 1 min at 94 °C (denaturation), 1 min at a specific annealing temperatures (Table 2) and 1 min at 72 °C (extension) followed by a final extension step at 72 °C for 5 min.

Amplifications were performed in a thermocycler (Biometra, Germany) for both RAPD and ISSR. At least two independent PCR amplifications were performed for each sample with RAPD and ISSR primers. DNA amplification fragments were separated in 1.8 % agarose gel using 1 $\times$ 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 1kb DNA ladder (Fermentas, Germany).

RESULTS AND DISCUSSION

A method of micropropagation through somatic embryogenesis in date palm was developed. This method involved development of embryogenic callus from shoot tips as well as primordial leaves. These 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. The resulting embryogenic callus (Fig. 1a) 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. The resulting matured somatic embryos (Fig. 1b) were germinated on a germination

medium for plant formation (Fig. 1c and 1d). 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) two PCR based techniques *viz*, RAPD and ISSR were used in 27 randomly chosen plants. Total 160 RAPD primers were screened, of which 30 primers which resulted in six or more scorable bands were selected for further study. These 30 RAPD primers generated 246 amplicons, ranging from 250 bp to 4000 bp in size. The number of bands in the selected primers varied from 6 (OPB 07, OPC 11, OPD 05, 07, 11, 12, OPF 12 and OPG 03) to 13 (OPA 15), with an average of 8.2 bands per RAPD primer (Table 1). In case of ISSR, 21 UBC primers

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

Table 1. List of different RAPD primers, primer sequences, and number of amplified bands and size range in the detection of genetic stability in micropropagated plants of date palm.

Primer	Primer sequence (5'-3')	No. of total bands	Size range (bp)
OPA-02	TGC CGA GCT G	10	400-2500
OPA-03	AGT CAG CCA C	8	250-4000
OPA-05	AGG GGT CTT G	8	300-1500
OPA-10	GTG ATC GCA G	9	400-2500
OPA-15	TTC CGA ACC C	13	400-2000
OPA-18	AGG TGA CCG T	7	300-2700
OPB-04	GGA GTG GAG T	11	250-700
OPB-07	GGT GAC GCA G	6	300-2100
OPB-10	CTG CTG GGA C	10	300-2400
OPB-17	AGG GAA CGA G	10	250-2000
OPC-05	GAT GAC CGC C	9	200-3000
OPC-08	TGG ACC GGT G	10	300-2000
OPC-11	AAA GCT GCG G	6	250-1500
OPD-02	GGA CCC AAC C	11	250-1800
OPD-05	TGA GCG GAC A	6	500-1500
OPD-07	TTG GCA CGG G	6	250-1500
OPD-11	AGC GCC ATT G	6	250-1000
OPD-12	CAC CGT ATC C	6	300-1200
OPD-18	GAG AGC CAA C	10	300-1200
OPE-01	CCC AAG GTC C	7	250-2500
OPE-15	ACG CAC AAC C	9	400-1700
OPE-18	GGA CTG CAG A	9	250-1500
OPF-10	GGA AGC TTG G	8	400-2000
OPF-12	ACG GTA CCA G	6	350-1200
OPG-02	GGC ACT GAG G	8	250-750
OPG-03	GAG CCC TCC A	6	300-1000
OPG-18	GGC TCA TGT G	7	250-1000
OPG-19	GTC AGG GCA A	9	300-1500
OPH-04	GGA AGT CGC C	8	250-2000
OPH-13	GAC GCC ACA C	7	300-2000

were screened and 12 primers which resulted in 6 or more scorable bands were selected for further study. These 12 ISSR primers generated 101 amplicons, ranging from 300 bp to 3000 bp in size. The number of bands for each primer varied from 6 (UBC 822, 824, 841 and 843) to 15 (UBC 834), with an average of 8.4 bands per ISSR primer (Table 2). A total of 8,592 bands (number of plants analysed x number of amplicons with all the primers) were generated by the RAPD and ISSR, and showed monomorphic pattern across all the 27 plants studied. A total of 30 RAPD and 12 ISSR primers generated 347 (246 RAPDs+101 ISSRs) distinct amplicons. These large numbers of amplicons being produced from two different markers, extended over distinct regions of the genome, are reasonably adequate for detecting somaclonal variations. This is evident by the comparable numbers of bands scored (326, 150 and 424) in various plant taxa by employing PCR-based marker assays (Martins *et al.*, 2004; Joshi and Dhavan, 2007; Lakshman *et al.*, 2007). An example of the monomorphic amplicons obtained for RAPD (Fig. 2a) and ISSR (Fig. 2b) markers are shown. No polymorphic bands were observed. 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 mode of micropropagation to produce true to type plants. There are many reports in literature, which cite similar results (Thakur *et al.*, 1999; Andrea *et al.*, 2004; Sharma *et al.*, 2004; Lopes *et al.*, 2006). Somatic embryogenesis is usually considered best next to axillary multiplication as there is stringent genetic control throughout the somatic embryo formation and the selection pressure against the abnormal types is considerably high (Leroy *et al.*, 2000). Other methods of regeneration such as organogenic differentiation are more prone to somatic variations (Vasil, 1987). Off-types have been detected from micropropagated plants in *Pinus thunbergii* (Goto *et al.*, 1998), pecan somatic embryos (Vendrame *et al.*, 1999), *Populus tremuloides* (Rahman and Rajora, 2001) and tea clones (Devarumath *et al.*, 2002). Hence, it becomes imperative to regularly check the genetic fidelity of the micropropagated plants in order to produce clonally uniform plants while using different techniques of micropropagation.

Extensive work has been done to facilitate the understanding of mechanisms leading to somaclonal variations, which include studies on chromosomal

Table 2. List of different UBC primers, primer sequences, GC content, annealing temp, number of amplified bands and size range, in the detection of genetic stability in micropropagated plants of date palm.

UBC Primer	Primer sequence (5'-3')	G+C content (%)	Annealing temperature (°C)	No. of total bands (bp)	size range
815	CTC TCT CTC TCT CTC TG	52.94	45	7	300-1500
818	CAC ACA CAC ACA CAC AG	52.94	45	7	350-1600
822	TCT CTC TCT CTC TCT CA	47.05	38	6	500-1200
824	TCT CTC TCT CTC TCT CG	52.94	45	6	450-1500
834	AGA GAG AGA GAG AGA G (CT) T	47.36	38	15	300-1500
835	AGA GAG AGA GAG AGA G (CT) C	52.63	44	11	350-2500
840	GAG AGA GAG AGA GAG A (CT) A	47.36	38	7	300-1500
841	GAG AGA GAG AGA GAG A (CT) C	52.63	44	6	400-1600
843	CTC TCT CTC TCT CTC T (AG) A	47.36	38	6	300-1500
846	CAC ACA CAC ACA CAC A (AG) T	47.36	38	9	500-1600
855	ACA CAC ACA CAC ACA C (CT) T	47.36	38	11	400-2500
888	(GCT) (AGT) (GCT) CAC ACA CAC ACA CA	52.17	43	10	300-3000

rearrangements and single gene mutations *etc.* (Phillips *et al.*, 1994). Somaclonal variations also occur as consequence of retrotransposon activation, which results due to stress conditions and lead to stable mutations.

Hirochika (1993) first reported the activation of retrotransposons leading to somaclonal variations due to tissue culture-induced stress in tobacco. Variations induced in tissue cultured plants likely to be reflected

Fig. 2. PCR amplification products obtained with (a) RAPD primer OPE-15 and (b) ISSR primer UBC-835. Lane M, represents one kb ladder; lane 1, represents mother plant; lanes 2-28, represent tissue cultured raised plants at the 42nd cycle of transfer.

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. Devarumath *et al.* (2002) revealed that ISSR fingerprinting detected more polymorphic loci (12.8%) than RAPD fingerprinting (4.28%) in micropropagated tea clones. The use of two types of markers, which amplify different regions of the genome, allows better chances for identification of genetic variation in the tissue culture raised plants. So, in the present study, we have adopted two PCR-based techniques *viz.* RAPD and ISSR, for the identification of somaclonal variation in micropropagated date palm plants.

CONCLUSION

In conclusion, this study has screened a large number of RAPD and ISSR primers that are common in higher plants and some of which are prevalent in monocots too. 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 somatic embryogenesis, can be effectively used for a considerable length of time under *in vitro* conditions (168 weeks) without much risk of genetic instability 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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