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RAPD analysis of Micropropagated Plantlets in Date Palm

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Abstract: Randomly Amplified Polymorphic DNA (RAPD) markers were used to analyze genetic stability of the somatic embryogenesis-derived regenerants (R_{1-6}) and mother plant in Iranian date palm (*phoenix dactylifera* L.) cultivar Khanizi. Total genomic DNA extracted from *in vitro* fresh leaves of regenerated plants and mother plant was amplified using 10-mer oligonucleotide Fermantas primers. Four primers of the set A to J primers were selected which revealed polymorphism and gave reproducible results. The genetic similarity between the mother plant and the callus-derived plantlets was ranged between 94% (for R_1, R_2) and 83% (for R_5). Cluster analysis by the unweighted paired group method of arithmetic mean (UPGMA) showed a single large cluster at an estimated similarity coefficient (90.2%).

Key words: Callus, *Phoenix dactylifera* L., RAPD-PCR, somatic embryogenesis

INTRODUCTION

Date palm (*phoenix dactylifera* L. Arecaceae) a long-living monocotyledon plant, is an important source of economic revenues for Iran and arid regions of the world. Due to dioecious and heterozygous characteristics of this species, it has to be propagated by vegetative means or else, it will result in heterogenous performances of seed derived palms[1,2]. Traditionally, date palm was propagated via offshoots that were produced by female trees during juvenile and adult phase. Limited numbers of offshots has made *in vitro* tissue culture an alternative method for mass propagation. For the past 20 years, date palm has been micropropagated by organogenesis[3] or somatic embryogenesis[4,5]. Currently, the process by somatic embryogenesis seems to be more attractive for industrial production but the date palm derived from somatic embryo must be true to type[1].

Due to somaclonal variation generated during *in vitro* culture, it cannot be certified that the genetic organization of tissue culture derived material is identical to the explant from which it originates and that it is inherently homogeneous[6]. The assessment of the genetic stability of *in vitro* derived clones is an essential step in the application of biotechnology for micropropagation of true to type clones[7]. Identification of off-types and genetically not identical to the mother plant at an early stage of development is considered to be very useful for quality control in plant tissue culture so the use of molecular markers is becoming widespread for the identification of somaclonal variants and the assessment of *in vitro* regeneration protocols[8]. RAPD markers have proved useful for the detection of genetic changes[2,8,9]. The aim of this study was to determine genetic changes between callus-derived plantlet, cv. Khanizi and mother plant of this Iranian date palm cultuar.

MATERIALS AND METHOD

Explant preparation: Offshoots from Khanizi cultivar were used in this study. They were collected from Minab in the south of Iran. Outer leaves of 3-4 years old offshoots were removed, exposing the shoot tip region about 8 cm long, this part was surface disinfected by immersion for 15 min in a 2.6% sodium hypochlorite solution with 1 ml L^{-1} Tween 20 and rinsed 4 times with sterile distilled water then shoot tip termini sectioned longitudinally into four pieces and put in culture medium.

Culture initiation and callus induction: The culture medium consisted of MS based salt medium[10] supplemented with 453 μM 2,4-D, 15 μM 2ip, 13 μM BAP. Cultures were incubated in darkness at 24±30°C for 5 months from which subcultured at 1 month intervals. The resultant embryogenic callus from each explant were transferred individually to callus proliferation medium that contained MS basal salt to which was added 5 μM NAA, 147 μM 2ip and 1.5 g activated charcoal. Callus proliferation and subsequent culture stages

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were maintained at 24±30°C, 16 h photoperiod (50 μmol m^{-2} s^{-1}) provided by cool-white fluorescent lamp. In proliferation medium calli[11-13] grew towards complete plantlets.

Plant genomic DNA extraction: Genomic DNA was extracted from *in vitro* fresh leaves of regenerated plantlets through somatic embryogenesis and fresh leaves of mother plant by CTAB method[14]. The nonionic detergent Cetyltrimethylammonium Bromide (CTAB) can be used to liberate and complex with total cellular nucleic acids from a wide array of plant genera and tissue types.

DNA amplification: All experiments were carried out twice for each primer after optimization of amplification reactions. Fermantas 10-mer oligonucleotide primers with GC contents of 50-60% were used for six regenerated plants and mother plant of date palm, cv. Khanizi. Total volume of 25 μL PCR reaction mixture contain 1 U of Taq DNA polymerase (Sinagene), 25 pM primer, 200 μM dNTPs, 200 μM $MgCl_2$, PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl, Sinagene) and 50 ng genomic DNA. Amplification were performed using Cyclogene Techne Termal Cycler with the following profile: (I) 94°C for 3 min x 1 cycle; (ii) 94°C for 45 sec, 36°C for 1 min, 72°C for 90 sec x 35 cycles; (iii) 72°C 5 minx 1 cycle. The amplification products were visualized on 1.4% (w/v) agarose stained with ethidium bromide. RAPDs of *in vivo* and *in vitro* material were compared using four selected primers that they were reproducible and the number of polymorphic bands was among 2-10[15].

RAPD analysis: Data were recorded as presence (1) or absence (0) of amplified products. Similarity coefficient ($S = 2x N_{ab}/N_a + N_b$) was calculated from band sharing data as described by Nei and Li[16] N_{ab} is number of bands shared by two lanes, while N_a and N_b represent number of bands present in lane a and b, respectively. Cluster analysis by the unweighted pair group method using arithmetic averages (UPGMA) was performed with the NTSYS pc-2.02e.

RESULTS

The availability of a suitable DNA isolation procedure is a prerequisite for performing DNA based markers studies on a date palm cultivar. CTAB method was chosen for this study. For confirming the reproducibility of the RAPD fingerprints for DNA/primer combinations, two independent DNA extracts prepared from the mother plant was tested for all primers. Similar banding paterns were observed for all the tested DNA/primers combination. Somaclonal variation was examined among 6 regenerated plants from embryogenic callus. A to J arbitrary 10-mer oligonucleotide sequences were screened for their amplifying ability using 7 date palm DNA samples. PCR amplification products obtained with the 6 somatic embryo-derived date palms were compared with that of their original mother palm. Four primers (A, B, E and H) successfully produced polymorphic RAPD profile for 6 regenerated palms (Table 1), for example, primer A produced 5 polymorphic non-parental bands. Among total of 57 amplified bands 25 bands were polymorphic (Table 2). The number of amplification products per primer ranged from 8 (E primer) to (H primer). Average of 14.3 RAPD markers was amplified per primer. The number of polymorphic bands per primer ranged from 4 (E primer) to (B primer). The

Table 1: Presence of non-parental polymorphic RAPD bands in date palm regenerants

Plants	P	R_1	R_2	R_3	R_4	R_5	R_6
Polymorphic bands							
A-1	–	–	–	–	–	+	–
A-3	–	–	+	–	–	–	–
A-4	–	+	+	+	+	+	+
A-6	–	–	+	–	+	+	–
A-10	–	–	+	–	–	+	–
B-2	–	+	+	+	+	+	+
B-7	–	–	–	–	–	–	+
B-10	–	–	–	–	–	+	–
B-13	–	+	+	+	+	+	+
E-4	–	–	–	–	–	+	–
E-5	–	–	+	–	–	–	–
E-8	–	–	+	–	+	+	+
H-7	–	–	–	–	–	+	–
H-16	–	–	+	–	–	–	–
H-17	–	+	–	–	–	–	–

P: mother plant; R_1-R_6: Regenerated plants; +: presence of a non-parental band; -: Absence of a non-parental band

Table 2: Names and Sequence of 4 primers that were selected for comparison between plantlets and mother plant of date palm

Primer	Sequence	Total bands	Polymorphic bands	Monomorphic bands
A	5′- GGT-CTC-CTAG-3'	12	6	6
B	5′- CGG-AGA-GCGA-3'	13	8	5
E	5′ - ACT-TGT-GCGG-3'	8	4	4
H	5′ - GGT-CAA-CCCT-3'	24	7	17
	Total	57	25	32

Table 3: Matrix of similarity coefficient based on RAPD profiles for 4 selected primers

	R_1	R_2	R_3	R_4	R_5	R_6	P
R_1	1.00						
R_2	0.89	1.00					
R_3	0.95	0.91	1.00				
R_4	0.95	0.93	0.97	1.00			
R_5	0.85	0.88	0.87	0.89	1.00		
R_6	0.92	0.90	0.94	0.93	0.86	1.00	
P	0.94	0.88	0.94	0.93	0.83	0.91	1.00

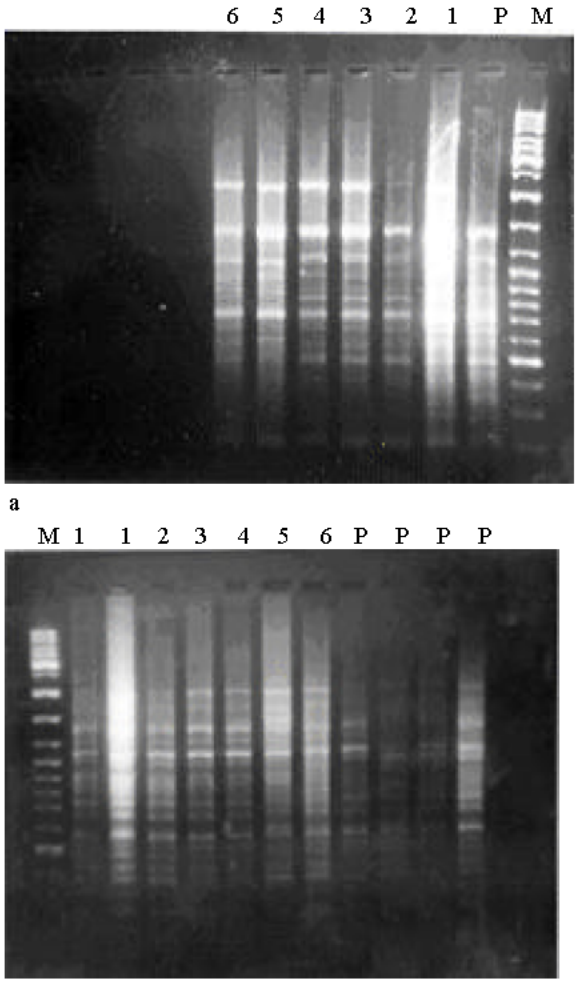

Fig. 1: RAPD PCR product of Primer B (a) and Primer H (b), callus-derived regenerants (1-6), mother Plant (p) and size Marker (M)

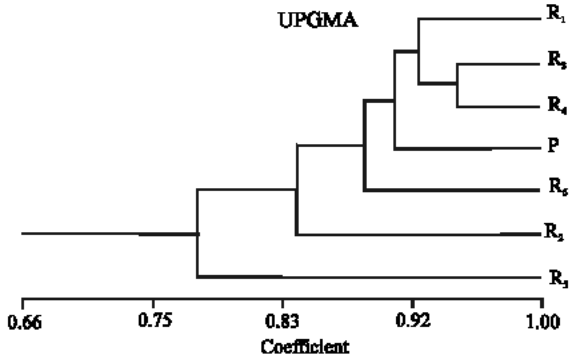

Fig. 2: Dendrogram of six Regenerants (R) and mother plant (P) of date palm (*Phoenix dactylifera* L.) by UPGMA cluster analysis based upon 4 RAPD primers. 6 regenerants formed a single cluster with mother plant

RAPD fingerprintings of 6 micropropagated palms and the mother palms for primer B and H has been shown in Fig. 1.

Genetic similarity was scored between regenerated plants and the mother plant by comparing their RAPD profiles for each of the four primers and calculating the coefficient of genetic similarity (Table 3) as described before.

Cluster analysis by the UPGMA showed all of the six micropropagated plants and the mother plant formed a single large cluster with an estimated average similarity coefficient with 90.2% (Fig. 2).

DISCUSSION

The aim of the present study was to provide polymorphic RAPD markers suitable for preliminary comparison between suckers and tissue culture derived plants from Iranian date palm cultivar Khanizi. The identification of variability in micropropagated plants derived from the same donor mother plant as in *Populus deltoids*[17] and *Piper longum*[18] using RAPD provides evidences for the existence of variants. Thus suggesting visual phenotypic evaluation may not be sufficient for characterizing the *in vitro* plants. Screening DNA variations among several millions of base-pair could be more problematic and exhaustive than scoring for a few morphological variations[9]. Since, even single base change at the primer annealing site is manifested as appearance or disappearance of RAPD bands, it could be suggested that tissue culture conditions can induce varied amount of genetic changes in different regenerated plants[19]. On the other hand, the observed small variations in DNA may have occurred de novo during the period of differentiated cell proliferation between the culturing of the explant and tissue regeneration[9]. Above mentioned changes appeared identical in different plants as regenerated by appearance of non-parental bands, for example A-6 (in R^2, R^4, R_5), B-2 (in R_1, R_2, R_3, R_4, R_5, R_6) and E-8 (R_2, R_3, R_4, R_5, R_6). Genetic variation in these plants could be because of they were all derived from the same callus[19]. Analysis of the coefficient of genetic similarity among the different plants indicated that all the callu-derived plantlets had varied degree of genetic difference from the mother plant as well as themselves. The genetic similarity between the mother plant and the callus-derived plantlets was high (average of 90.2%) and ranged between 94% for R_1, R_3 and 83% for R_5.

Evidently, R5 had accumulated maximum genetic changes (as represented by presence of 10 non-parental RAPD bands in this plant) and had lowest average coefficient of genetic similarity (83%) compared to other regenerated plants. The *in vitro* regeneration process was shown to be mainly responsible for the RAPD banding difference among the plantlets.

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