In Vitro Culture and Genetic Analysis of Male and Female Date Palm (*Phoenix dactylifera* L.)

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Abstract

Previously for micropropagation of date palm only shoot tip was used as an explant. In an attempt to utilize different explants, shoot tip and base of flower rachis of male and female plants of variety 'Barhy' were used to initiate callus cultures on modified Murashige and Skoog medium containing different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D), 2-isopentenylaminpurine (2-iP), kinetin and α-naphthalene acetic acid (NAA). Male shoot tip cultures produced embryogenic callus at significantly lower frequency as compared to the female cultures. Female rachis cultures produced embryogenic callus while male rachis cultures produced mostly non-embryogenic callus. After two years, only female shoot tip cultures produced plantlets. Liberation of phenolics was significantly higher in male cultures, enabling differentiation between male and female plants and justifying the slow growth of male cultures.

For early identification of cultivars and tracing genetic diversity among date palm genotypes of different origin, offshoot-derived, male and female plants of cultivars 'Barhy' and 'Sukkary', seed-derived plants, and two in vitro cultures of both of these cultivars were subjected to Randomly Amplified Polymorphic DNA (RAPD) analysis. Similarity matrixes based on Nei and Li's coefficients show that offshoot-derived male plant of 'Barhy' was 73.6% genetically similar to its female counterpart, while similarity between male and female plants of 'Sukkary' was 43.1%. In the case of seedlings, male and female plants of 'Barhy' were 87.2% similar and those of 'Sukkary' were 62.3% genetically alike. Two in vitro cultures of 'Barhy' and Sukkary were 73.3% and 70% similar to the normal offshoot-derived plant respectively. These affinities were also reflected in cluster analysis by unweighted paired group means (UPGMA). Results of the study suggested that DNA fingerprinting can be utilized for early detection of date palm sex and genotype and that in vitro behavior of male and female plants of date palm cultivars is differential and their DNA profiles are divergent, which can help in selecting preferable males.

INTRODUCTION

With its ability to accumulate exceptionally high level of metabolites under extreme arid conditions, date palm (*Phoenix dactylifera* L.) is a unique physiological entity. The species is well adapted to desert regimes and constitutes the nucleus of biodiversity in these inhospitable areas. For centuries it has been one of the most important crops in many agriculturally desolate regions of the tropics and arid regions including Saudi Arabia.

Date palm is a dioecious species and its seed is generally heterozygotic, giving rise to heterogeneous progeny. For plantation purpose, therefore, only vegetative offshoots of preferred genotypes are used to ensure genetic uniformity. Multiplication rate is exceedingly low through this process and supply of true-to-type planting material is always far below the demand in date palm growing areas. Lately, micropropagation through tissue culture was used in Saudi Arabia for generating large populations of date palm plants at a rapid pace (Al-Khalifah, 2000; Al-Ghamdi, 1993). For commercial reasons, however, these efforts are confined to the female plants of cultivars in demand in respective areas.

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Considering the occurrence of metaxenia and differential varietal compatibility in the species (El-Ghayati, 1983), using pollen of appropriate genotype in right quantity is imperative for optimizing yield and quality of the fruit. For the same reason, procedures must be developed for in vitro production of male date palm plants. In seed-derived populations, half of the plants are expected to be males and half of the females diverse from there genetical origin. Early identification of these plants is essential for maintaining only the required number of male plants in a plantation and for cultivar selection.

A date palm tree produces limited number of offshoots in its life span. Offshoots are the sole source of variety perpetuation, production of in vitro-derived plants being in miniscule, and are therefore, a scarce commodity. Using explants other than shoot tip for initiating in vitro cultures in date palm is highly desirable especially in the preferred qualities that have no or less off-shoots.

Some degree of somaclonal variation is known to occur in tissue culture-derived populations of various plant species (Karp, 1995). The phenomenon has been reported in date palm also (Al-Wasel, 2001; Azeqour et al., 2002), this is considered to be a disadvantages due to the minimization of true to type plants . Early detection of variations in tissue culture-derived plants by DNA finger printing techniques is highly needed as it is helpful in eliminating undesirable genetical variation.

Genotype identification in date palm is an intricate empirical exercise based on morphological characters (Sedra et al., 1998; Bashah, 1996). In most cases, identification can be done only after fruiting, which takes place after 5-7 years. Hence discrepancies in naming of cultivars are believed to be prevailing widely in this species (Bennaceur et al., 1991). Randomly Amplified Polymorphic DNA (RAPD) is a comparatively simple, quick and less expensive procedure for generating genomic markers (Welsh & McClelland, 1990; Williams et al., 1990). The technique has been successfully used for varietal identification in date palms (Al-Khalifah and Askari, 2003; Askari et al., 2003).

In this study, in vitro response of male and female explants, i.e. shoot tip and flower rachis of cultivar 'Barhy' has been compared. RAPD analysis of male and female plants of offshoot and seed origin of cultivars 'Barhy' and 'Sukkary', and two in vitro cultures of each of these cultivars was carried out in order to early detect and figure out differential genotypic profiles.

MATERIALS AND METHODS

For initiating in vitro cultures, shoot tips were excised from 2-3 year old offshoots of male and female plants of cultivar 'Barhy'. Segments of the very soft part of rachis, 2-3 cm in length, were obtained from just opened inflorescence. The explants were sterilized with sodium hypochlorite solution (1.0%) for 20 minutes, followed by mercuric chloride solution (0.1%) for 5 minutes and were rinsed 4-5 times with sterilized distilled water.

All media contained salts, organic supplements, vitamins and sucrose as per MS medium (Murashige and Skoog, 1962) and were gelled with Agar (7.5 g/L); pH of the media was adjusted to 5.8 before autoclaving. Three replicate jars of 5 explants in each were maintained in a completely randomized design (CRD). Experiment was repeated and data was collected from two triles.

For callus induction, explants were placed on a medium supplemented with 100 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 3.0 mg/L 2-sopentenylaminopurine (2-ip) and 3.0 mg/L Kinetin. To control browning of cultures, activated charcoal (1.5 g/L). ascorbic acid (75 mg/L) and citric acid (75 mg/L) were added to the medium.

Three to four weeks after callus induction, primary callus was transferred to another medium for development of embryogenic callus. Hormone supplement of the medium was: 2.5 mg/L α-naphthalene acetic acid (NAA), 3.0 mg/L 2-ip and 3.0 mg/L Kinetin. The callus was sub-cultured on the same medium every 6-7 weeks for further production of embryos. Cultures were incubated in growth rooms at 26°C +1 and light intensity of 2500 Lux with a photo period of 16 hours light. Well-developed embryos were shifted to hormone-free medium for multiplication and shoot formation.

Production of phenolic compounds in culture was estimated according to Shetty et

al. (1995) and was expressed in µg/g dry weight of the tissue. Statistical analysis applied to compare different explants performance in vitro.

For RAPD analysis, genomic DNA was extracted from fresh young leaves, of offshoot-derived and seedling derived male and female plants of cultivated varieties of 'Barhy' and 'Sukkary' (Genotypes of the male seedlings were not confirmed, these were based on expectations of the farmers). Materials were also taken from two in vitro grown female cultures of these cultivars. Extraction was done according to the procedure of Dellaporta et al. (1983). Yield of DNA was measured by Hoefer DyNA Quant 2000 (Pharmacia Biotech) and the concentration in TE buffer was adjusted to 25 $ng.\mu^{-1}$. Polymerase Chain Reaction (PCR) amplification procedures were followed according to Al-Khalifah and Askari (2003). Overall, 13 primers of A and B series (Operon Technologies Inc., USA) were used for PCR.

RAPD products were separated by electrophoresis on 1.4% agarose gel submerged in 1x TBE buffer. DNA profiles were figured and documented by Gel Doc-2000 system (Bio Rad).

DNA polymorphs were scored as present (1) or absent (0). The data was analyzed using software, 'Diversity Data Base' (Bio Rad). Genetic similarities between the genotypes were estimated on the basis of shared amplification patterns (Nei and Li, 1979). Dendrograms were constructed on the basis of similarity coefficients by using unpaired group of arithmetic means (UPGMA).

RESULTS

Overall, in vitro performance of male cultures was significantly inferior to female cultures (Table 1). Shoot tip cultures of both, male and female plants showed superior response than rachis cultures (Figs. 1.a-d). Callus induction frequency in male shoot tip and rachis cultures was 35.5% and 8.9% respectively as compared to 57.7% and 17.8% in the corresponding female cultures. Only 20% of the male shoot tip calli showed embryogenic activity as compared to 35.7% of the female calli. None of the male rachis calli turned out to be embryogenic, while 6.7% of the female rachis calli were embryogenic. Male shoot tip cultures took 16 months to develop embryogenic callus while embryogenic calli developed in female shoot tip cultures in 6 months.

Highest secretion of phenolics was observed in male rachis cultures (15.50 µg/g dry weight), followed by female rachis (6.65 µg/g), male offshoot (2.43 µg/g), and female offshoot cultures (0.80 µg/g). A significant negative correlation was noticed between production of phenolics and callus initiation (Table 1).

Yield of DNA from different samples ranged from 10 to 30 ng/mg fresh weight of tissue. In each sample, different primers produced varying degrees of polymorphism of the DNA (Fig. 2).

Six samples of 'Barhy' revealed ample polymorphism (Fig. 2a). Genetic similarities between different samples of 'Barhy' ranged from 67.4% to 87.2% (Fig. 3a). Offshoot-derived male 'Barhy' was 73.6% similar to its female counterpart, while seed-derived male and female of this variety were 87.2% similar. This result confirms assigning of this male to 'Barhy'. Two in vitro cultures of 'Barhy' were 73.3% and 68.4% matching to normal offshoot-derived female 'Barhy'. Cluster analysis (Fig. 4a) shows that offshoot-derived male and female plants of 'Barhy' are placed apart, whereas seed-derived male and female plants fall in the same cluster. Both in vitro cultures are showing discreteness from offshoot-derived female genotype.

Polymorphism was detected in the case of 'Sukkary' also (Fig. 2b). Genetic similarities ranged between 37.7% and 88.9% (Fig. 3b). DNA profile of male 'Sukkary' of offshoot and seed-derived origin matched 47% with the female genotype (Fig. 4b). This observation suggests that males, which were believed to be 'Sukkary' by the farmers, may not belong to this variety. Two juvenile cultures of 'Sukkary' are showing 70.6% genetic similarity with offshoot derived female plant.

Dendrogram (Fig. 4b) of 'Sukkary' showed lower affinity between offshoot-derived male and female genotypes than their seed-derived counterparts. Tissue culture

plants showing a high mutual relationship are placed far away from offshoot-derived female plant.

DISCUSSION

Production of somatic embryos in the cultures of male shoot tip explant of date palm is an important observation of this study. Establishment of regeneration procedures in male cultures would provide a scope for generating adequate number of male plants of choice and will provide selected male material.

Attempts have been made earlier to induce callusing in different explants of date palm including rachis. Sharma et al. (1980) tried different explants and used several media but no positive response was elicited in these experiments. Bhaskaran and Smith (1992) were able to regenerate plants using shoot tip and immature inflorescence as an explant. Development of embryogenic callus in female rachis cultures, despite low culture response, is a significant step towards finding an alternative to the scarce and expensive shoot tip explant of selected trees especially aged once that no longer produce off shoots.

Browning of the explant due to secretion of phenolics is a serious problem in date palm cultures (Zaid, 1984). In the present study, differential behavior of male culture from both, shoot tip and rachis explants in liberating significantly higher levels of phenolics compared to female cultures is conspicuous. It was noticed that a significant negative correlation existed between developmental responses in vitro and production of phenolics in cultures. Male cultures from both explants produced higher levels of phenolics and showed correspondingly lower frequency of callus induction and embryogenesis. Highest value of coefficient of correlation (r) was observed for male rachis cultures, which showed the least callusing response. On the contrary, female offshoot cultures with the least secretion of phenolics gave the highest response. Excessive production of phenolics in male rachis cultures could be the cause of absolute mortality of these cultures. Browning in date palm cultures could be reduced by trimming the explants to a smaller size and sub-culturing calli at short intervals, especially in the cultures from rachis explants. The amount of phenolic compounds can be used to distinguish between the male and female at early stages of growth of date palm plantlets.

High levels of polymorphism have been reported in date palm earlier (Al-Khalifah and Askari, 2003; Askari et al., 2003). Mokhtar et al. (2000) have observed that cultivars similar in morphological characters tend to cluster together in RAPD based dendrograms. In the present study, polymorphism expressed in the PCR products was of a magnitude, which should allow differentiation between the samples, enhancing the scope for utility of RAPD technique for genotype differentiation in date palm.

Sedra et al. (1998) attempted to differentiate between the two sexes in date palm cultivars by RAPD procedures and concluded that their materials were genetically too similar to be differentiated on the basis of available profiles. Similarity level between male and female plants appears to be variable among cultivars.

Closer genetic affinity between male and female seedlings of 'Barhy' may be because these were the first generation plants from the seeds of the same mother plant. Male and female plants of 'Sukkary' may not be related due to different sources of seeds. Divergence between the profiles of male and female plants of 'Sukkary' supports the possibility of utilizing DNA finger printing for differentiating between the sexes of date palm genotypes, which is considered important for establishing good quality plantations.

In vitro grown cultures 'Sukkary' showed a close genetic similarity between themselves because they were from the same mother plant. On the other hand, cultures of 'Barhy' showed low similarity because they were derived from different mother plants.

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Tables

Table 1. In vitro response of male and female cultures of date palm cv. 'Barhy'.

Genotype/ Explant	Callus initiation (%)	Embryogenic calli (%)	Time to initiation of embryogenic calli (months)	Production of phenolics (µg/ g DW)	Correlation (r) phenolics-callusing
Male shoot tip	35.5	20.0	16	2.43	-0.745*
Female shoot tip	57.7	35.7	6	0.80	-0.709*
Male Rachis	8.9	0.0	-	15.50	-0.913**
Female Rachis	17.8	6.7	8	6.65	-0.802**
LSD $_{P\geq 5\%}$	11.7	7.4	-	3.2	-

Figures

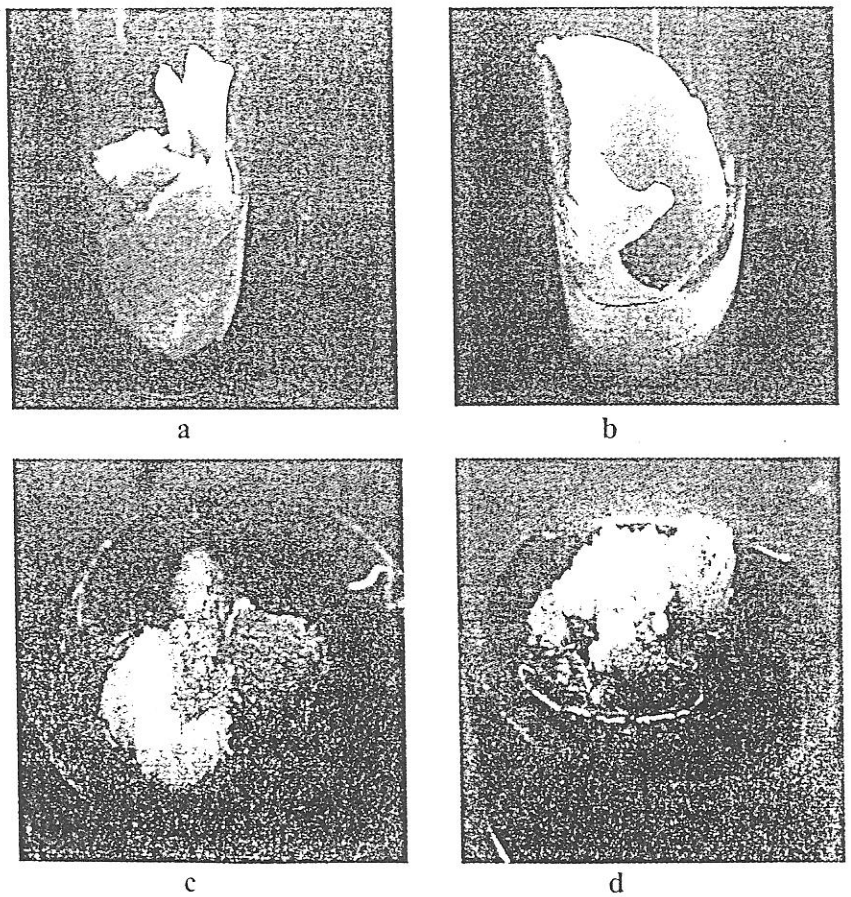


Fig. 1. In vitro response of male and female cultures of date palm cv. 'Barhy'. (a) Browning and degeneration of male shoot tip explant. (b) Initial callus formation in male shoot tip cultures. (c) Non-embryogenic callus in female rachis cultures. (d) Embryogenic callus in male shoot tip cultures.

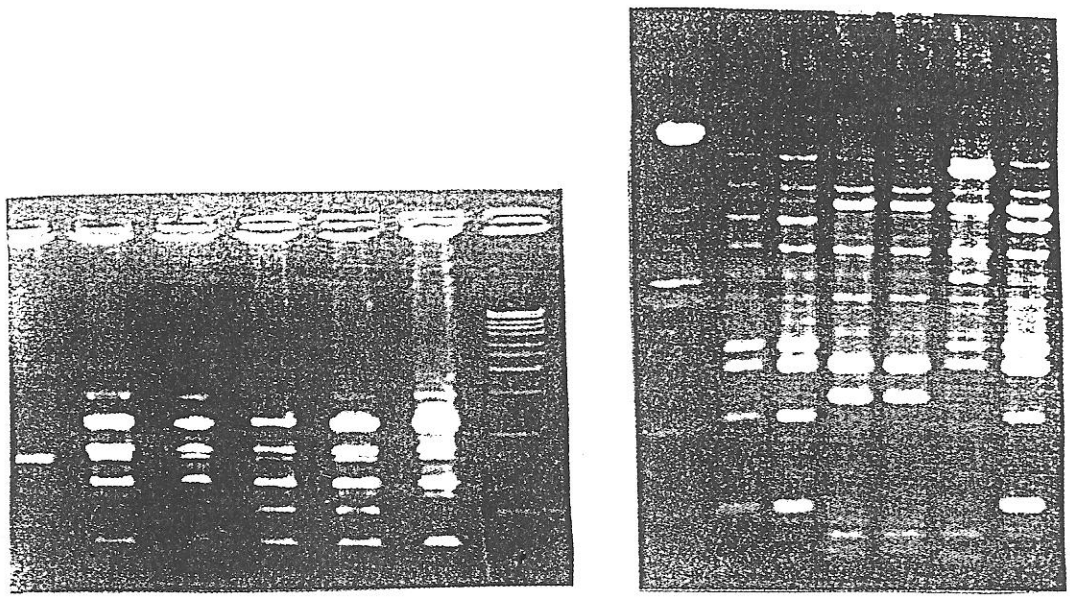


Fig. 2. RAPD profiles of two date palm cultivars. (a) var. 'Barhy', primer: B10, (b) var. 'Sukkary', primer: A7. M = Marker (kb ladder). 1 = Offshoot-derived female, 2 = in vitro culture-1, 3 = in vitro culture-2, 4 = Offshoot-derived male, 5 = Seed-derived female, 6 = Seed-derived male.

(a) var. 'Barhy'

		1	2	3	4	5	6
Bar Female OD	1	100.0					
Bar Female SD	2	73.1	100.0				
Bar Male OD	3	73.6	82.7	100.0			
Bar Male SD	4	69.8	87.2	76.3	100.0		
TC-1	5	68.4	70.8	75.6	67.4	100.0	
TC-2	6	73.3	80.4	85.1	76.0	77.4	100.0

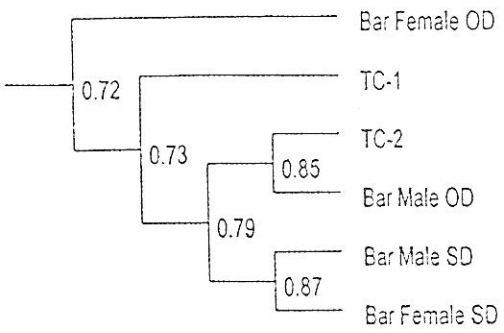
(b) var. 'Sukkary'

		1	2	3	4	5	6
Suk Female OD	1	100.0					
Suk Female SD	2	70.0	100.0				
Suk Male OD	3	43.1	57.6	100.0			
Suk Male SD	4	41.5	62.3	69.4	100.0		
Suk TC-1	5	70.6	76.2	41.5	47.3	100.0	
Suk TC-2	6	70.6	71.4	37.7	43.6	88.9	100.0

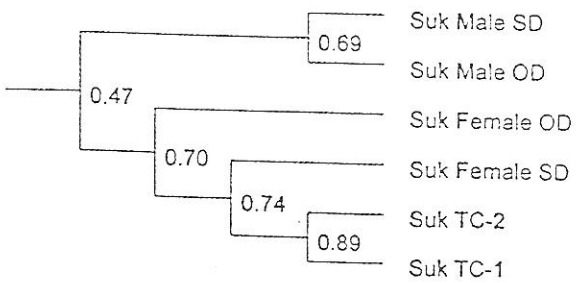
Fig. 3. Similarity matrices of DNA from two cultivars of date palm based on Nei and Li's coefficient.

Fig. 4.

(a) var. 'Barhy'



(b) var. 'Sukkary'



and Li's

Fig. 4. Dendrograms of genetic relationship between samples of two cultivars of date palms.