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GENETIC AND ANATOMICAL ANALYSIS OF NORMAL AND ABNORMAL FLOWERS OF DATE PALM CULTIVAR 'BARHY' DERIVED FROM OFFSHOOT AND TISSUE CULTURE

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Abstract

Random Amplified Polymorphic DNA (RAPD) analysis between 6 normal flower producing offshoot derived and 6 abnormal multiple carpel, flower producing tissue culture (TC) derived trees of cultivar (cv.) 'Barhy', was performed with the objective to check genetic variation if any at DNA level. DNA samples were extracted from pollinated and un-pollinated flowers from both sets of plants. Amplified RAPD products were clearly detected with 30 primers used in this experiment but only 3 gave a few polymorphic bands which shows low level of genetic variation among the offshoot and TC derived plants. Cluster analysis by the unweighted paired group method of arithmetic means (UPGMA) showed close genomic similarity among the 12 DNA samples with the range of 0.486-0.904 Nei and Li's coefficient in the similarity matrix. The average similarity among the 12 DNA samples was more than 50%. Floral abnormalities in TC derived plants were also studied microscopically. Abnormalities like more than three carpel development, abnormal ovule development and deformities of style and stigma were observed. The results show that the composition and the abnormalities of flowers in TC derived plants of cultivar 'Barhy' may be attributed to epigenetic changes that takes place at different stages of tissue culture and not due to major changes at DNA level.

Key words: *Phoenix dactylifera*·RAPD ·epigenetic ·embryogenesis ·fruit abnormality flower abnormality.

Introduction

Since long history date palm (*Phoenix dactylifera* L.) is considered as most consumed fruit tree in Middle East and North of Africa. Saudi Arabia is one of the countries of the Middle East that produces and exports the best quality dates of the world (Zaid & de Wet, 1999). In Saudi Arabia alone 450 cultivars are found (Bashah, 1996). The techniques of date palm tissue culture (TC) have recently been introduced and the development of it made possible massive expansion cultivars but commercially TC propagated plants were introduced only in the last few years. The main cultivars are Hayani, Bentaisha, Barhi, Zahedi, Ameri and Halawy. Because of the difficulty associated with morphological identification of date palm cultivars, development of cultivar specific genetic markers have been extensively studied (El Kichaoui *et al*., 2013).

The authors (Al-Khalifah *et al.*, 2006; Al-Khayri, 2003; Tisserat, 1982, 1979; Zaid & de Wet, 1999; Bhansali *et al.*, 1988) claimed that Propagation either through offshoots or TC techniques like somatic embryogenesis results in true-to-type plants. However, off-types that are not identical to their progenitor are also produced due to somaclonal variation caused by genetic and or epigenetic changes during TC process (Kunert *et al*., 2003, 2002; Kaeppler *et al*., 2000; Sala *et al*., 1999; Cullis *et al*., 1999). In TC derived date palm many off-type phenotypes are detected that include stunted or severely retarded growth, leaves with wide leaflets, variegated leaf, malformation of inflorescence, abnormal flowers and low levels of fruit setting (Gurevich *et al*., 2005; Cohen *et al*., 2004; McCubbin *et al*., 2000). Some of these abnormalities in many trees become normal after 8-10 years of plantation and are therefore believed to be due to epigenetic changes (Cohen *et al*., 2004). Epigenetic changes are expressed under stress conditions possibly due to DNA methylation, DNA amplification and or activation of transposable elements (Kaeppler *et al*., 2000; Brar & Jain, 1998; Hirochika *et al*., 1996; Brettell & Dennis, 1991).

Abnormal flowers and low levels of normal fruit setting, especially in TC plants of cultivar Barhy, are common in Saudi Arabia. Large numbers of tissue cultured plants introduced in the country in 1995 are producing an average of more than 60% abnormal flowers and fruitlets per plant causing low levels of fruit set which is of a great economic loss to the farmers (Kunert *et al*., 2003). A reasonable of low levels of fruit setting may be inadequate pollination due to abnormalities in the flowers (carpels, style and stigma). As a consequence all the three unfertilized carpels develop into abnormal and small fruitlets (local name *Shees*). In normal date flowers, after pollination, only a single carpel develops into a fruit and the other two carpels degenerate. More than three carpelled florets and fruitlets (4-7 carpels) are also observed (Gurevich *et al*., 2005; Cohen *et al*., 2004; Al-Wasel, 2001; Djerbi, 2000; Reuveni, 1986). It is possible that the supernumerary carpels other than the main three carpels are staminodialprimordia transformed into carpel-like structures (Cohen *et al*., 2004). Almost the same abnormalities have been observed in flowers of TC derived oil palms (Corley *et al*., 1986).

Molecular genetically analysis of TC plants showing abnormal phenotypes was performed by using RAPD (Saker *et al*., 2000) and isozymes (Azeqour *et al*., 2002). The molecular techniques detected plantlets that were showing difference in the traits at TC stage but were unable to resolve differences in the abnormal traits that appeared later at mature stage of the plant growth. Gurevich *et al*. (2005) and Askari *et al*., 2003) have recently performed AFLP analysis of both normal and off type (low fruit setting) TC derived plants of cv. Barhy but were unable to detect any AFLP marker linked to abnormal flowering and low level of fruit setting traits.

In the present study RAPD analysis, between normal flower producing offshoot derived and abnormal multiple carpel, flower producing TC derived trees of cv. 'Barhy', was performed with the objective to see genetic variation if any at DNA level. Floral abnormalities in TC derived plants were also studied microscopically.

Material and Methods

Total genomic DNA extraction: Total genomic DNA was extracted from 10 fresh flowers of each of the 12 samples.

Fresh flowersfrom12 different plants; 6normal flower producing offshoot plants and 6 multiple carpel flower (*shees*) producing TC derived plants, were collected from an orchard near Al-Kharj area of Saudi Arabia (Table 1). Flower samples were taken ten days before and after the pollination from both true-to-type offshoots and off-type TC plants.

Table 1. List of 12 plants originated from offshoot and or tissue culture that were used in this study.

Accession #	Mode of cultivation	Phenotype
N-14/1 (O)	Offshoot	Normal
N-14/3 (O)	Offshoot	Normal
N-15/4 (O)	Offshoot	Normal
N-14/1 (C)	Offshoot	Offshoot
N-14/3 (C)	Offshoot	Offshoot
N-15/4 (C)	Offshoot	Offshoot
P-R2A1G1 (O) (1)	Offshoot	Multiple carpel flowers
P-R2A1G1 (O) (3)	Tissue culture	Multiple carpel flowers
P-R2A2G1 (O) (6)	Tissue culture	Multiple carpel flowers
C-R2A2G1 (C) (1	Tissue culture	Multiple carpel flowers
C-R2A2G1 (C) (3)	Tissue culture	Multiple carpel flowers
C-R2A2G1 (C) (6)	Tissue culture	Multiple carpel flowers

The samples were first ground into a fine powder in liquid nitrogen by using pestle and mortar and then by following the steps of the protocol developed by Dellaporta *et al*. (1983). Pure and highly intact DNA was extracted. The quality and quantity of the DNA was determined by using a fluorometer (Hoefer DyNA Quant 200, Pharmacia Biotech). The integrity of the DNA was also determined by agaroseminigel electrophoresis.

Primers and PCR amplification: A total of 30 RAPD primers of A, B, C, and D series (OPERO Tech., CA, USA) were used for polymerase chain reaction (PCR) amplification of the 12 different DNA templates to construct DNA fingerprinting profiles. PCR amplification was performed as described by Al-Khalifah & Askari (2003). The RAPD products of each primer was separated by electrophoresis according to their molecular weight in 1.4% agarose gels submerged in 1 x TBE buffer and then stained with ethidium bromide (10 µg ml^{-1}) solution for 20 minutes. The DNA were visualized on UV-transilluminator and documented with Gel Documentation System (Bio Rad). The molecular weights of the amplified fragments were estimated by running Kilo Base DNA marker (Amersham Pharmacia Biotech.) in gel as standard size marker.

Genetic analysis: The amplification profiles of the 12 different 'Barhy' samples were compared with each other by using software 'Diversity Data Base' (Bio Rad). The data was applied to estimate the genomic similarity among the plants on the basis of shared amplified fragments (Nei, 1978; Nei& Li, 1979). Cluster analysis by the unweighted paired group of arithmetic means (UPGMA) was also performed and a dandrogram was constructed by Dice Coefficient Method with the help of the software 'Diversity Data Base.

Morphology and microscopy of flowers: Ten to twenty fresh flowers each from six different offshoots and six different TC plants were fixed in FAA (10% formaldehyde, 5% acetic acid, 50% ethanol). The flowers were collected ten days before and 40 days after the pollination. Dehydration and embedding of fixed materials were done in paraffin wax. Thin transverse and longitudinal sections of flower buds were cut at 5 µm thickness on Reichert-Jung rotary microtome and permanent slides were prepared following Johansen (1940). The sections of flowers were examined under a compound microscope and images were documented with a digital camera. The shape and structure of carpels and stigmas were studied to detect abnormality.

Discussion

Genetically analysis of normal and abnormal fruit bearing plants: RAPD analysis was performed on the 30 RAPD profiles of the 12 DNA samples of 'Barhy' that include 6 normal fruit bearing offshoot derived plants and 6 abnormal, multiple carpel, fruit bearing TC derived plants with the aim to assess genetic variation if any exist among them. The 30 primers used in this experiment were prescreened and selected for date palm DNA fingerprinting. The average similarity among the 12 samples of DNA was more than 50%, suggesting close genomic similarities between the samples. Maximum similarity was observed between offshoot derived plants (N 15/4 C) with normal unpalliated flowers and TC derived plant (P-R2A2G1 6) with multiple carpel pollinated flowers (0.90). Offshoot derived plant (N 14/1 C) with normal unpalliated flowers showed 88% genomic similarity with them which is the second highest similarity value. Offshoot derived plant (N 14/3 O) with normal pollinated flowers and TC derived plant (P-R2A1G1) with multiple carpel pollinated flowers are more related genomically (0.86)

while TC derived plant (C-R2A1G1) with multiple carpel unpollinated flowers is 76% gnomically similar with them. Similarly offshoot derived plant (N 14/1 O) with normal pollinated flowers and TC derived plant (P-R2A2G1 3) with multiple carpel pollinated flowers are also genomically similar with similarity matrix value 0.81. TC derived plant with multiple carpel unpollinated flowers in general showed a minimum degree of similarity with all the other samples ranging between 0.48-0.72. Saker *et al*. (2000) have detected low level of genetic variation in young TC derived plantlets (4% of 70 analyzed plantlets) by using RAPD markers. Only those plantlets that were phenotypically different at TC level were detected as genetically variant. Gurevich *et al*. (2005) recently applied AFLP on normal offshoots and abnormal flower and fruit bearing TC derived plants of cv. 'Barhy' detecting low level of genetic variation among the plants but were unable to link any AFLP marker to this abnormal flower and fruit setting character. We can therefore suggest that the abnormal flower and fruit bearing trait in TC derived plants of cv. 'Barhy' are due to the epigenetic changes that occur during the TC stages, and not due to any major genomic changes. Most of the abnormal fruit bearing TC plants become normal after 8-10 years of flowering but in some plants increase in the percentage of abnormal fruits also occur indicating epigenetic effects that are expressed under stress conditions during tissue culture. The stress conditions may cause DNA methylation, DNA amplification and or activation of transposable elements in the plants (Kaeppler *et al*., 2000). Kichaoui AE *et al*., 2013 applied Random amplification of polymorphic DNA (RAPD) to study the genetic diversity among the six cultivars using 42 primers. They constricted genetic similarity matrices for the six cultivars using the Nei and Li formula and clustered with the UPGMA to determine the relationships between the six cultivars. In our study limited number of plant DNA samples and RAPD primers were used. It could be one of the reasons for detecting low level of genetic variation and not detecting any linked RAPD marker to this particular phenotype. RAPD markers should be of high value for assessing the genetic variance in date palm. It is therefore desirable to increase the size of the population and also the number of RAPD primers to better assess the genetic diversity and detect linked markers to this phenotype.

Results

The results of genetic variations suggest that RAPD analysis could be efficiently used for the assessments of genetic variation. Although low level of DNA polymorphism was observed, some genetic variation was detected between offshoot and TC derived plants. Amplified RAPD products were clearly detected with all the 30 primers but only 3 produced reproducible and distinguishable polymorphic bands which indicate low level of genetic variation among the offshoot and TC derived plants (Fig. 1a & b). The pair-wise genetic similarity was estimated for the 12 samples of DNA on the basis of Nei & Li's (1979) similarity coefficients (Table 1). A similarity matrix was also constructed (Table 2). Cluster analysis by the unweight paired group of arithmetic means (UPGMA) method showed close genomic similarity among the 12 DNA samples with the range of 0.486-0.904 Nei and Li's coefficients in the similarity matrix. A dandrogram by using Dice coefficient method for the 12 genotypes of Barhy by using 'Diversity Data Base software' (Bio Rad.) is presented in Fig. 2. The number of polymorphic bands per primer varied from 3 to 6, with a mean of 3 major bands per primer. The present RAPD data so far generated suggest narrow genetic diversity among the offshoot derived normal flowering plants and TC derived abnormal flowering plants indicating that most of the plants of cv. 'Barhy' are true-to-type and have nearly the same genetic makeup. Flowers and fruits in plants propagated from offshoots and TC originated orchards were observed (Fig. 3a-e). Low fruit setting has close relationship with structural floral abnormalities in TC derived plants such as abnormal development of carpel, stigma and ovule (Fig. 3c & e). A longer twisted stigma attached with narrow carpel head rather than clear joint between stigma and carpel was observed (Fig. 3c). Because of these abnormalities pollen tube loses its path that leads to failure of fertilization. Same results were also found by different workers during their studies. Our observation revealed that many TC derived plants have major fruit setting problems. Beside normal single fruits many abnormalities were recorded such as single carpel parthenocarpic fruits, parthenocarpic fruits with two or three carpels originated from tri-carpellary flowers (Fig. 3a & d) and abnormal fruit lets with additional supernumerary carpels (Fig. 3a).

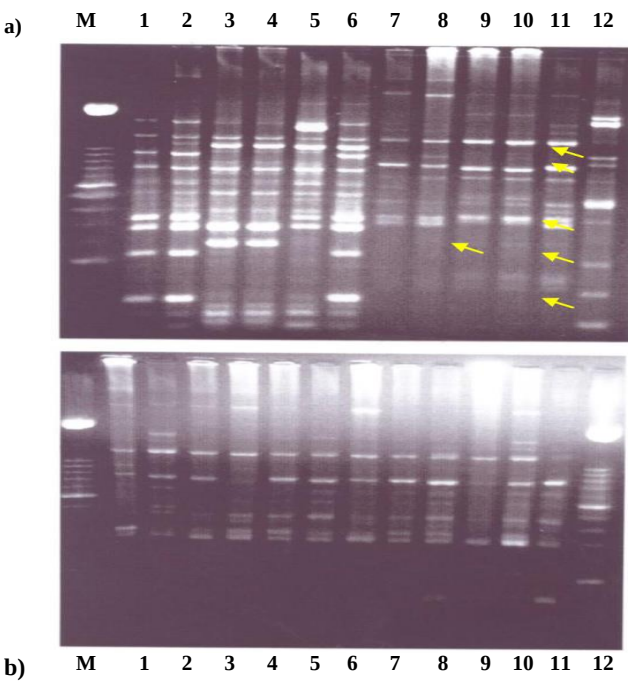

Fig. 1. RAPD profiles of 6 multiple carpel flower (shees) producing TC derived plants and 6 normal flower producing offshoot plants of cultivar 'Barhy' using OPA7 (a) and OPB16 (b) primers. Lanes: M (Molecular weight markers) 1, 2, 3, 4, 5 & 6 multiple carpel flower (shees) producing TC derived plants. Lanes: 7, 8, 9, 10, 11 & 12 normal flower producing offshoot plants.

Table 2. Similarity matrix for Nei and Li's coefficients of 6 normal flower producing offshoots and 6 multiple carpel flower (shees) producing TC derived genotypes of cultivar 'Barhy' obtained from RAPD markers.

		1	2	3	4	5	6	7	8	9	10	11	12
14/3(C)	1	100.0											
C-R2A2G1(6) (C)	2	81.9	100.0										
N15/4	3	79.5	77.5	100.0									
N15/4 (C)	4	85.7	81.0	78.4	100.0								
N-14/1 (C)	5	81.1	71.6	76.1	88.0	100.0							
N-14/1 (C)	6	52.9	61.3	55.4	55.1	51.5	100.0						
N-14/3 (O)	7	63.6	63.0	60.3	68.7	56.3	75.9	100.0					
P-R2A1G1 (O)	8	62.3	52.9	58.6	67.7	57.6	64.2	86.3	100.0				
P-R2A1G1 (O)	9	58.7	70.7	52.8	65.8	57.7	80.6	80.0	70.0	100.0			
P-R2A2G1	10	83.3	75.9	72.5	90.4	88.6	50.0	61.3	66.7	59.2	100.0		
R2A1G1 (C) (1	11	62.3	52.9	51.7	58.1	54.0	79.2	74.5	78.3	70.0	59.6	100.0	
R2A1G1 (C) (3	12	60.3	65.0	48.6	59.5	50.7	70.8	69.8	58.6	72.2	52.2	62.1	100.0

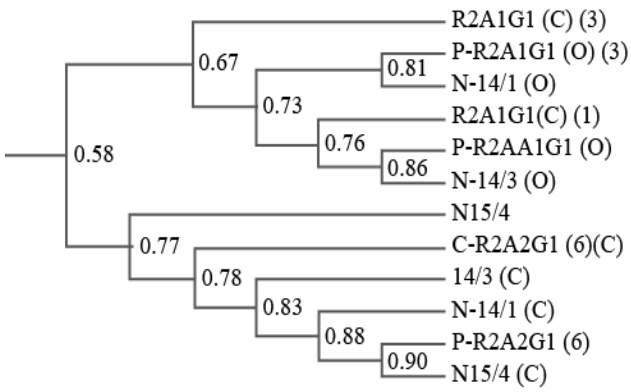

Fig. 2. A dendrogram of phylogenetic relationships among 6 normal flower producing offshoots and 6 multiple carpel flower (*shees*) producing TC derived genotypes of cultivar 'Barhy' based on Nei and Li's similarity coefficients generated from 30 RAPD profiles.

Fig. 3. Fruit morphology and microphotographs of date palm flowers from offshoot and TC derived plants. a. A comparison of the normal and multi-carpel fruits. b. Longitudinal section (LS) of closed flower of offshoot derived plant showing normal development of carpel and ovule. c. LS of open flower of TC plant showing abnormal stigma and style in both carpel and abnormal ovule in only one carpel. d. Transverse section (TS) of closed flower of TC plant showing development of all the three carpels and ovules that lead to shees formation. e. TS of closed flower of TC plant showing abnormal development of two carples without any ovule.

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