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LEAF CHARACTERISTICS AND DNA ANALYSIS OF IN VITRO REGENERATED PLANTLETS OF DATE PALM (*Phoenix dactylifera*, L.)

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ABSTRACT

The in vitro regenerated plantlets of date palm (*Phoenix daetylifera* L.) cv. Sewi, Via somatic embryogenesis, were visually screened at an early stage of their development; one foliage leaf. The observed off-types were classified into nine morphological groups and were compared to the normal plantlets with respect to morphological, anatomical characteristics of the leaf in addition to DNA profile using RAPD-PCR technique. The morphological variations included petiole shape and length, lamina shape, length, width and colour, whereas the main anatomical changes included mesophyll thickness, midrib zone, number of vascular bundles and xylem elements. DNA analysis revealed a high level of genetic variation correlated with the morphological changes.

INTRODUCTION

Date Palm (*phoenix dactylifera*, L.) is a monocotyledonous dioecious tree species, of major economic importance in the arid regions of the Middle East Gulf area and North Africa (Al-Khayri, 2002). It is considered on of the most important fruit trees cultivated in Egypt (about 7 million trees) and other Middle East and Arab countries (Mohamed, 1983; Moursy and Saker, 1998).

The cultivated grown cultivars are used for food, fiber, fuel and shelter (Al-Bakr, 1972; Zohary and Spiegel-Roy, 1975). Date palm is tolerant to drought, high temperature and salinity (Bouchreib and Clarck, 1997), therefore it is very promising crop to cultivate in new reclaimed soils in Egypt. Moreover, some cultivars were imported to Egypt from the Gulf area for their super fruit qualities. Therefore, the need for modern multiplication tools which insure the mass production of true-to-type plants became very necessary to satisfies the market demand (Moursy and Saker, 1998). The conventional method of vegetative propagation using offshoots is slow and expensive due to the limited number of off-shoots growth per plant (Pareek, 1984). The use of tissue culture techniques via indirect somatic embryogenesis or organogenesis in clonal propagation of data palm has been established in recent years as a routine procedure in several commercial laboratories to produce large numbers of date palm plants at a competitive cost (Kunert et al., 2003). Indirect somatic embryogenesis via proliferation of embryogenic callus is currenty the most efficient technique regarding its high rate of multiplication. This process of propagation requires the application of relatively high levels of auxin-type plant growth regulator (2, 4-D) (Tisserat, 1979, Bhaskaran and Smith, 1995). However, these auxins known to be associated with genetic instability in plants, a phenomenon called somaclonal variation (Karp, 1989; Phillips et al., 1994; Cullis, 1999). Although somaclonal variation can be used as a source for variation to obtain superior clones, it

can be also a very serious problem resulting in the production of undesirable plant off-types (Karp, 1993; Cassells et al., 1999). Several types of abnormalities were observed through the in vitro derived palm trees including variegation of leaves dwarfism, compact growth with broader leaves, leaf variegation, different spine structures, bending of stems, delayed flowering time, failure of pollination, abnormal fruiting, seedless fruits and higher susceptibility to diseases (Al-Sakran, 2001, Zaid and Al Kaabi, 2003; Mc. Cubbin et al. 2004). Most of these variations can affect plant productivity and cause massive financial losses for farmers. Therefore, observation and exclusion of these variants at early stages of propagation is of great importance.

The present study aimed to identify the morphological and anatomical characteristics of the off-types plantlets at early stage of their development and to study DNA fingerprints of the in vitro produced plantlets of the local date palm (Sewi) cultivar.

MATERIALS AND METHODS

This work was performed at the Laboratory of Tissue Culture, Agricultural Botany Department, Faculty of Agriculture, Cairo University, Giza.

The technique of in vitro plantlet regeneration via somatic embryogenesis of date palm cv. Sewi was adopted according to Abd El-Hamid, et al.,(2001). Three different explants, shoot apex (SA), axillary bud (AB) and leaf primordia (LP) were cultivated on MS medium containing 30 mg/L 2,4-D and 20 mg/L 2, ip for callus induction and proliferation and formation of somatic embryos. For further embryo development and germination, embryos were transferred to auxin – free medium. At the stage of one foliage leaf the plantlets were visually screened and the observed off-types were classified into nine categories (A-I) according to the morphological characteristics of the leaf . The leaves were morphological described. Petiole length, lamina length and width were measured, colours of lamina and petiole were recorded.

For anatomical study, specimens of cm^2 from the middle zone of the leaf lamina were killed and fixed in F. A. A., washed in 50% alcohol, dehydrated in normal butyl and embedded in paraffin wax (55°C m) Sass, 1958. Cross sections, 20 μ thick, were cut and stained by crystal violet/erythrosin combination and mounted in Canada balsam (Jackson 1926). Slides were microscopically examined. Measurements of different tissues were taken and averages of 10 readings from 5 slides were calculated. The coefficient of variation (CV) for all morphological and anatomical characteristics of leaves were estimated.

DNA Fingerprinting:

Random sample were taken from leaves of mother plant, and in vitro regenerated plantlets which originated from different types of explants, shoot apex (SA), axillary bud (AB) and leaf primordia (LP) . DNA extraction and RAPD - PCR techniques were performed according to Mc Garvey and Kaper (1991).

Data analysis for the genetic similarity coefficient (Gs) was estimated according to Dice coefficient, (Sneath and Sokal, 1973).

RESULTS AND DISCUSSION

Morphological studies:

Shape and morphological measurements of leaf petiole and lomina are presented in Fig. (1) and Table (1).

Concerning the shape of the leaf petiole, normal leaf and all abnormal leaves (off-types) had a cylindrical straight petiole except in off – types A, C and I the petioles were twisted, slightly twisted and with twisted edges; respectively. As for the petiole length, the average lengths of the off-types ranged between 0.2 to 0.3 cm; compared with 1.0 cm for the petiole of the normal plantlet leaf. The off-type A has the longest petiole (3.0 cm) white the off-type G has the shortest one 0.2 cm.

Table (1): Measurements of types of normal plantlet (CP) and off-types (A-I) leaves of date palm cv. Sewi of in vitro regenerated (averages of readings).

Types of the leaf / Characters	Normal Plantlet (control) cp	Off types								
		A	B	C	D	E	F	G	H	I
Petiole length (cm)	1.0	3.0	1.3	0.4	1.2	1.0	0.3	0.2	0.5	0.6
Lamina length (cm)	7.0	3.7	4.4	2.5	2.0	1.7	1.3	2.0	2.3	2.0
Lamina width (cm)	0.3	0.3	0.8	0.4	0.7	0.5	0.4	0.3	0.3	0.8

The petiole colour for all off-types was white compared with green petiole of the normal leaf. As for lamina shape, the normal plantlet has long, narrow and thin lamina, the lamina shape was widely varied on different off-types categories (A-I); it was long, cylindrical with narrow expanded distal half (A); long, broad and thick (B); long, cylindrical, thin and spirally twisted (C), short, broad and thick (D); long, broad , thick with twisted distal half (E); short, cylindrical, thick and curved (F); short, cylindrical, curved with twisted (G); medium, cylindrical and thick (H); medium, broad, very thick with curved end (I).

The averages of lamina length of all off-types were lower than that of the normal plantlet, they ranged between 1.3 cm and 4.4 cm compared with 7.0 cm of the normal plantlet.

Concerning the width of lamina, the averages for off-types ranged from 0.3 cm to 0.8 cm, compared with 0.3 cm for the normal plantlet. The off-type B and I had the widest lamina (0.8 cm), while the off types A, G and H had the narrowest lamina and recorded the same value (0.3 cm).

Concerning to the lamina colour, it was found that, lamina colour in off-types A, F, G, H and I was white; in D and E was green yellowish; in B was green; while in C was pale green; compared with the green colour for the lamina of the normal plantlet.

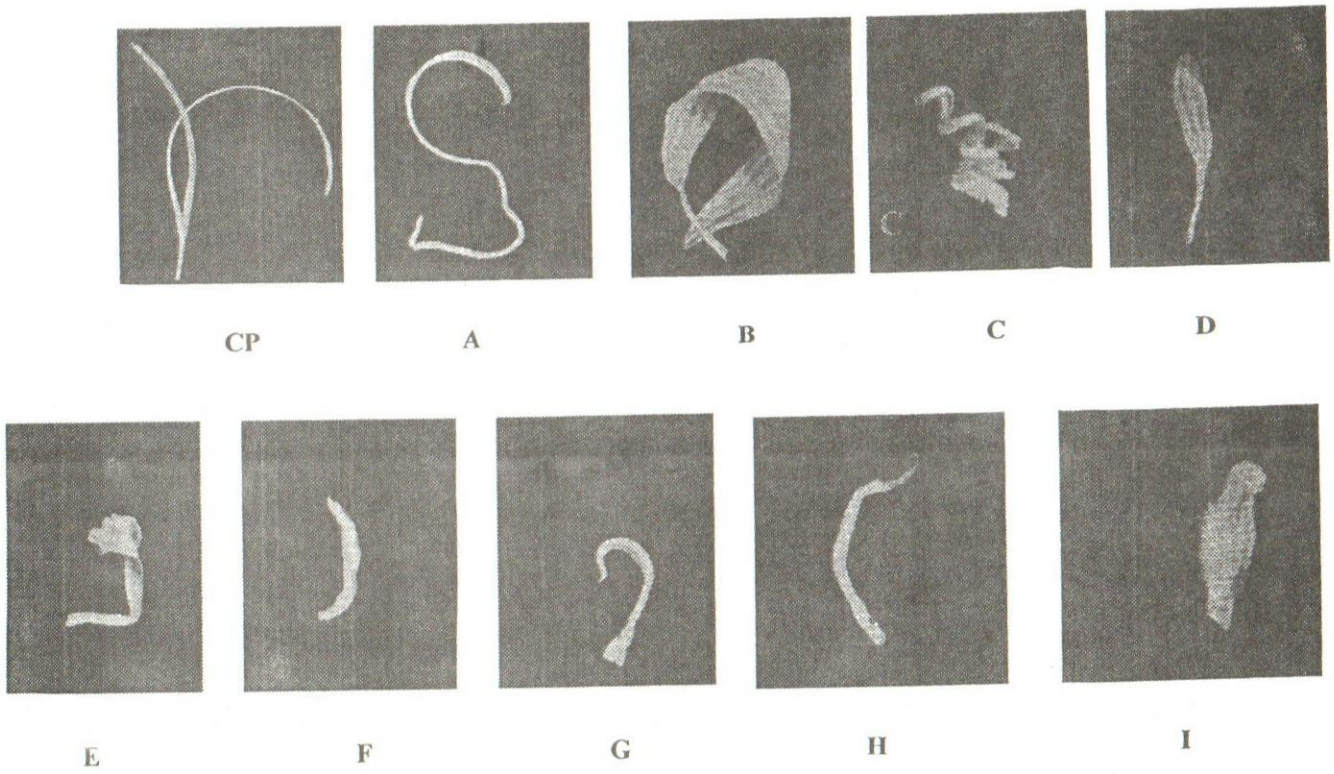


Fig. (1) : Shape categories of first foliage leaf of in vitro regenerated plantlets; normal (control plant) (CP); off-types (A-I).

The phenotypic variability of plantlets at the early stage of clonal propagation can be easily observed and excluded from successive steps of propagation. In previous work (Abd El-Hamid et al., 2001) the percentage of somaclonal phenotypic variants observed in vitro reached 4-6%, through somatic embryogenesis devied plants of cv. Sewi depending on the explant used. Mc Cubbin et al. (2004) stated that the frequency of abnormalities observed through field verification of palm trees of eight cultivars generated through somatic embryogenesis reached about 5%.

Zaid and Al Kaabi, (2003) reported that variations observed on in vitro plants included dwarfs, un proportniate shoot : root ratio, Sheath restriction on leaf emergence and blind meristem where the shoot meristem appears to be blocked or resricted.

Values of the coefficient of variation (CV) indirect that the highest degree of variability 86.3% recorded for petiole length followed by 59.5% for lamina length and the lowest value 32.3% for lamina width.

Anatomical structure of the leaf:

Cross sections in the first foliage leaves of the normal and off-types plantlets are shown in Fig. (2). Measurements of the main anatomical features of those leaves are presented in Table (2). It is obvious that, there were no differences between upper and lower epidermis thickness in all off-types and the normal plantlet (cp); it was nearly 14 μ thick.

In general the midrib zone was easily defined in the normal leaf and in some off-types; A, B, C, D and E; while in the other off-types it was difficult to define this region. It was observed that in the midrib zone of some off-type leaves, there were more than 3 randomly arranged vascular bundles with different sizes. It was clear that, the off-type (C) has the thicker mesophyll (772.8 μ) compared with (227.2 μ) for the normal plantlet. The average measurements for the mesophyll thickness were between 196.0 μ to 772.8 μ; and the off-type E has the lowest mesophyll thickness.

Concerning the vascular tissues it was found that the average number of vascular bundles in most off-types (36 to 111 bundles) was higher than that of the normal plantlet (27 bundle) except in off – types H and I where the numbers were 23 and 26 respectively.

On the other hand the normal leaf had higher averages to comparing with leaves of all off-types with respect to thickness of large bundle (290.2 μ) vs (173 to 288 μ) and width (201.4 μ) vs (103. to 148.4μ).

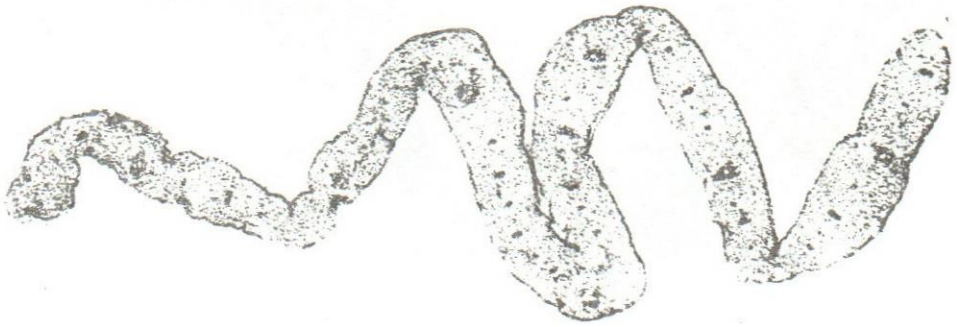
The thickness of xylem in the large bundle was (159.2 μ) vs (61.6 μ to 103.6 μ); and phloem thickness was (62.4 μ) vs (36.4 μ to 50.4 μ).

Small bundle thickness (135.0 μ) vs (49.0 μ to 134.4 μ) and its width was (88.7 μ) vs (60.2 μ to 92.4 μ) except off-type A where the bundle width was 92.4 μ.

While the thickness of phloem and xylem in small bundles were lower in normal leaf (24.2 μ and 43.6 μ) respectively than in off-types leaves vs (24.5 μ to 35.0 μ) and (30.8 μ to 60.2 μ) respectively;; except off-types B, F, G and H which had thinner xylem.

Table (2): Measurements of certain microscopical features in transvers sections in leaves of regenerated plantlets of date palm cv. sewi (Averages of 10 readings).

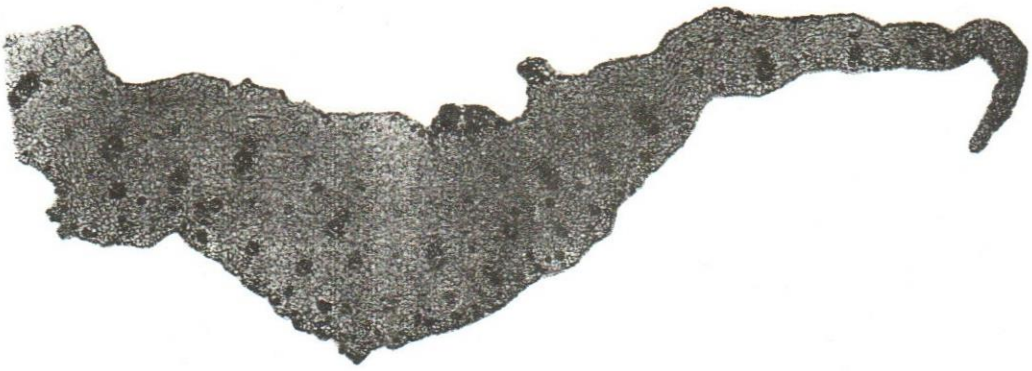
Characters / Types of the leaf	Normal plantlet	Off-types								
	CP	A	B	C	D	E	F	G	H	I
Thickness of mesophyll (μ)	227.2	238.0	535.5	772.8	363.4	196.0	431.2	564.0	481.0	572.8
Thickness of the large bundle (μ)	290.2	175.0	177.8	173.6	288.4	179.2	231.0	231.0	235.2	276.5
Width of the large bundle (μ)	201.4	126.0	109.2	106.4	148.4	103.6	143.5	140.0	112.0	133.0
Thickness of xylem in the large bundle (μ)	159.2	70.0	61.6	78.4	103.6	86.8	91.0	84.0	78.4	94.5
Thickness of phloem in the large bundle (μ)	62.4	37.8	39.2	36.4	50.4	39.2	42.0	42.0	36.4	45.5
Thickness of the small bundle (μ)	135.0	81.2	49.0	126.0	123.2	109.2	92.4	107.8	129.5	134.4
Width of the small bundle (μ)	88.7	92.4	84.0	77.0	78.4	61.6	72.8	60.2	66.5	84.0
Thickness of xylem in the small bundle (μ)	43.6	56.0	35.0	59.5	56.0	44.8	30.8	37.8	30.8	60.2
Thickness of phloem in the small bundle (μ)	24.2	35.0	24.5	28.0	30.8	28.0	28.0	28.0	28.0	28.0
Number of bundles of the lamino	27	58	49	48	111	46	64	36	23	26



CP

A

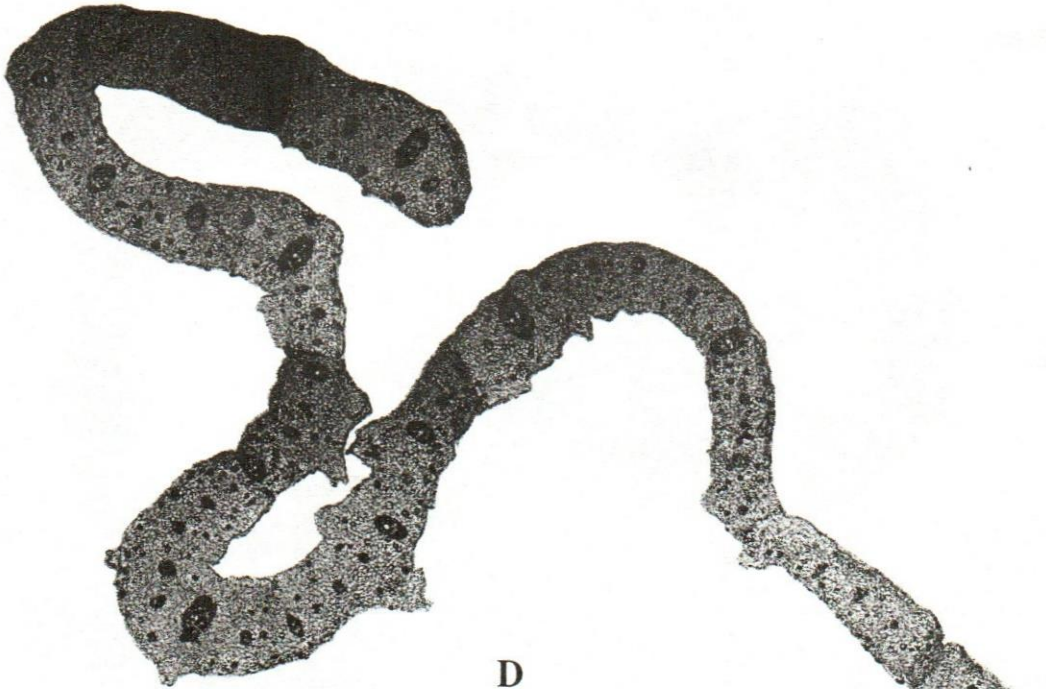
Fig. (2): Transaction in leaves of in vitro regernerated plantlets of date palm:
(cp) control of plantlet leaf (X 24.7)
(A) off – type leaf (X 5.9)



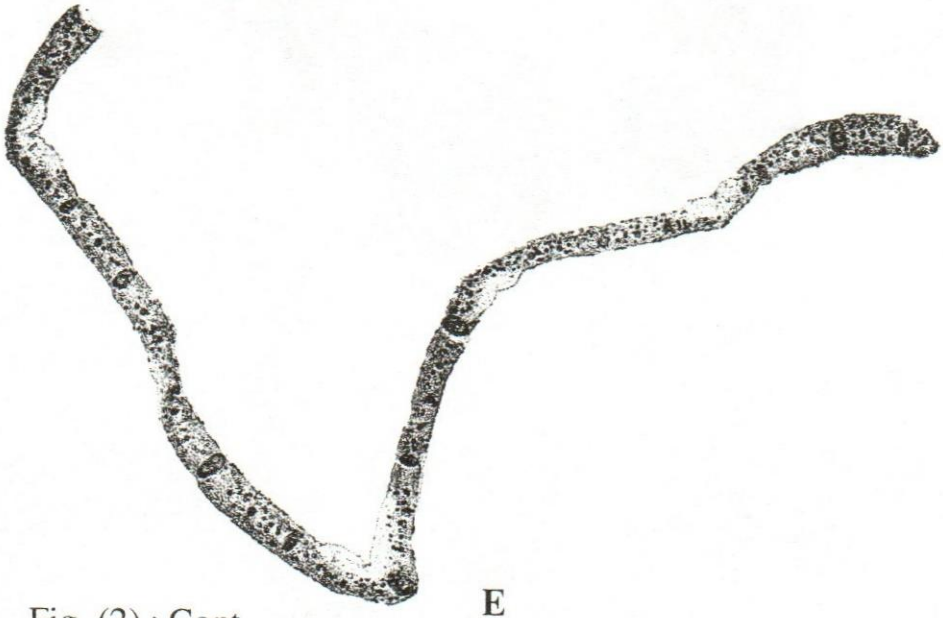
B

C

Fig. (2) : Cont.
(B) off – type leaf (X 11.7)
(C) off – type leaf (X 15.11)

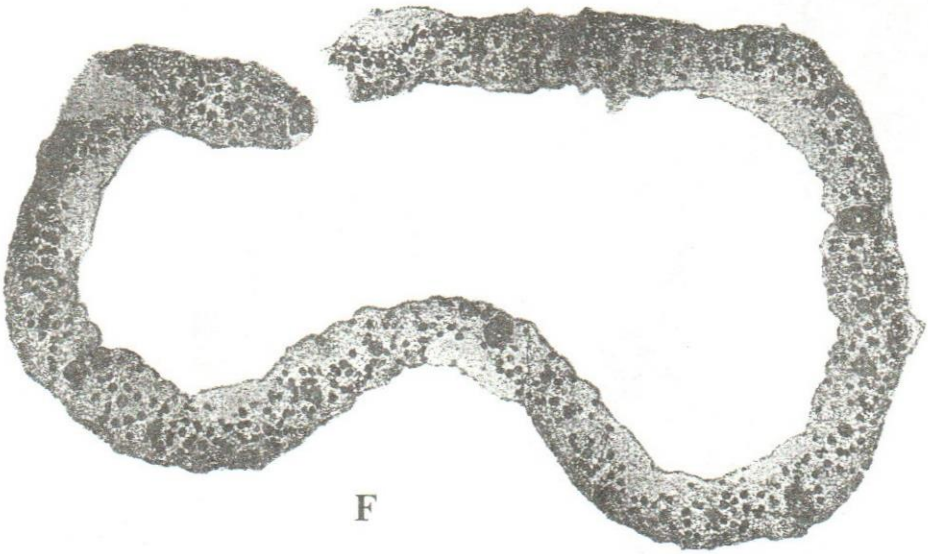


D

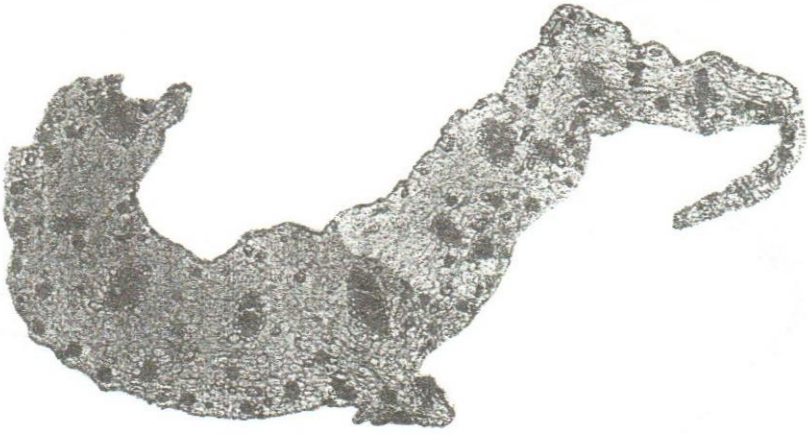


E

Fig. (2) : Cont.
(D) off – type leaf (X 9.8)
(E) off – type leaf (X 8.2)



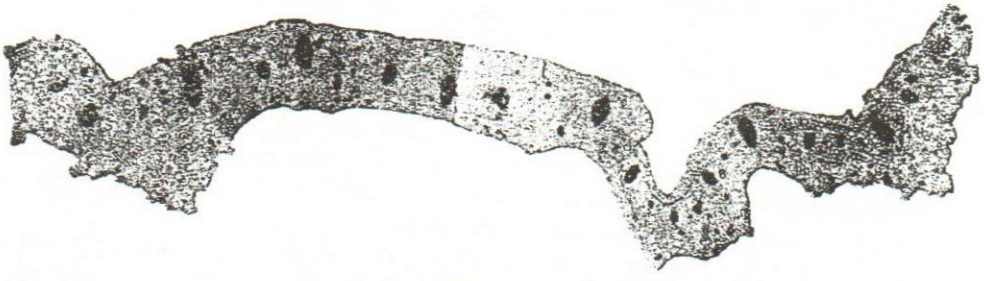
F



G

Fig. (2) : Cont.
(F) off – type leaf (X 8.9)
(G) off – type leaf (X 13.5)

H



I

Fig. (2) : Cont.
(H) off – type leaf (X 10.4)
(I) off – type leaf (X 4.9)

Regarding the values of coefficient of variation of anatomical measurements, it is evident that the highest value of variation was recorded for mesophyll thickness (61.1%) followed by that of number of bundles (52.99%). The variation values in xylem tissues 29.64% in large bundle and 35.38% in small bundle were higher than that of phloem; 18.65% and 10.74% respectively.

It could be concluded that the increase in thickness of the mesophyll tissue in leaves of most off-types was accompanied with an increase in the number of vascular bundles. However these vascular bundles were smaller in size with reduced vascular tissues especially xylem, comparing with those of the leaf of the normal plantlet.

DNA fingerprinting:

DNAs extracted from leaves of the mother plant and plantlets regenerated from shoot apex, axillary bud and leaf primordia were used for DNA fingerprinting using RAPD-PCR technique.

In preliminary survey, 5 primers were used for evaluating their ability to produce PCR amplification of date palm genomic DNA, from these primers as shown in Table (3), it was found that primer OPB-15 gave the best and clear bands with the tested samples.

Table (3): Primers used in RAPD analysis

Primer	Sequence (5` - 3`)	GC %
OPB 07	GGTGACGCAG	70
OPB 12	CCTTGACGCA	60
OPB 15	GGAGGGTGTT	60
OPB 17	AGGGAACGAG	60
OPB 18	CCACAGCAGT	60

Genetic similarity as revealed by RAPD marker OPB-15

Data in Table (4) and Fig. (3) represent the molecular weights of all tested samples by RAPD primer OPB-15 and total number of resolved bands. As total of 10, 12 and 14 bands were identified for the plantlet developed from SA, AB and LP respectively; compared with 8 bands for the mother plant. In molecular weights for the plantlets bands ranged from 445 to 2540 bp, while it ranged from 510 to 2540 bp for the mother plant.

To determine the genetic similarity among the regenerated plantlets and the mother plant, the scoring data (1 for presence and 0 for absence) resulting from the tested primer (Table 4) were used to compute the similarity matrices.

As shown in Table (5), the highest genetic similarity with the mother plant (80.3%) was recorded for plantlet regenerated from the shoot apex (SA) comparing with 67.5% and 59.3% for axillary bud (AB) and leaf primordia (LP) plantlets respectively.

Table (4): Total bands and molecular weights of the tested samples; plantlets developed from shoot apex (SA); axillary bud (AB); leaf primordia (LP) and mother plant (MP) according to PAPDs.

Total bands	Molecular weights	Samples			
		SA	AB	LP	MP
1	2540	1	1	1	1
2	2322	1	0	1	0
3	2027	0	1	1	1
4	1574	1	1	1	1
5	1355	1	1	1	1
6	1078	1	1	1	1
7	981	1	1	1	1
8	872	1	1	1	0
9	759	1	1	1	1
10	661	1	1	1	0
11	603	0	1	1	1
12	510	1	1	1	0
13	508	0	0	1	0
14	445	0	1	1	0

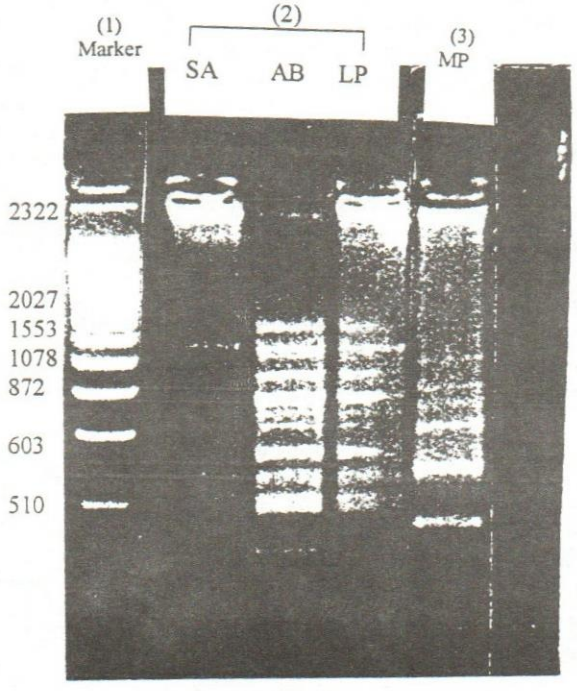


Fig. (3): RAPD profile for individual date palm samples;
(1) Marker
(2) Leaves of plantlets developed from; shoot apex (SA); axillary bud (AB) and leaf primordia (LP)
(3) Mother plant (MP)

The results of genetic similarity indicate the high level of genetic variation among the regenerated plantlets regardless to the type of explant from which they were derived. In this respect Saker et al. (2000) analyzed tissue culture-derived from date palm plants using RAPD analysis and showed that genetic variations occurred in approximately 4% of the analyzed plants, they suggested that somaclonal variation is due to the high level of 2, 4-D used in the protocol of somatic embryogenesis.

The technique of RAPD analysis also enalbled to differentiate between different cultivars of date palm, (Corniquel and Merciet 1994); (Saker et al. 1998). RAPD analysis is normally found to be easy to perform but has the major disadvantage that reproducibility is difficult to achieve between different laboratories and often even between different people in the same laboratory (Jones et al., 1997). Moreover, RAPD analysis covers only an extremely small portion of the genome, therefore it is difficult to verify whether any mutative variation is ultimately applicable for detection of true-to-type plants with this technique (Zaid and Al-Kaabi, 2003). The present results indicate that somatic embryogenesis as a protocol of date palm microprogation is not completely safe with respect to the true – to – type ness of the regenerated plants. Micropropagation of date palm via organogenesis especially from male and female inflorescences is supposedly easier to manage and might also lower the risk of plant off-type production (Loutfi and Chlyah, 1998).

In conclusion, it could be started that the morphological variations of leaves of some plantlets regenerated via somatic embryogenesis; off – types, are mostly expected to be correlated with variations at the anatomical and genomic levels. Therefore, it could be recommended that exclusion of those phenotypic variants at the early stages of micropropagation could decrease the frequency of somaclonal variation in palm trees derived from somatic embryogenesis.

Table (5): Genetic similarity (GS) matrices compouted according to Dice coefficient from RAPDs for in vitro regenerated plantlets and mother plant

Samples	In vitro plantlets			Mother plant
	SA	AB	LP	
I- **Plantlets from:**				
1) Shoot apex (SA)	100	71.4	71.4	80.3
2) Axillary bud (AB)		100	85.7	67.5
3) Leaf primordia (LP)			100	59.3
II- Mother plant				100

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صفات الورقة وتحليل الـ DNA للنبتات المتكشفة معمليا من نخيل البلح

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تم ملاحظة النبتات المتكشفة معمليا من الأجنة الجسدية من نخيل البلح (*Phoenix dactylifera* L.) صنف سيوى فى المراحل المبكرة من تطورها (الورقة الخضرية الأولى). صنفت النبتات الشاذة التى شوهدت إلى ٩ مجموعات مورفولوجية وقورنت مع النبتة الطبيعية مع الرجوع إلى الصفات المورفولوجية والتشريحية للورقة بالإضافة إلى تحليل الـ DNA باستخدام تكنيك RAPD-PCR. شملت الأختلافات المورفولوجية شكل العنق وطوله، شكل النصل وطوله وعرضه ولونه بينما شملت التغيرات التشريحية سمك الميزوفيل ومنطقة العرق الوسطى وعدد الحزم الوعائية وعناصر الخشب. أشار تحليل الـ DNA على وجود درجة كبيرة من الأختلافات الوراثية المرتبطة بالتغيرات المورفولوجية.