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ORIGINAL ARTICLE

Genotoxicity assessment of high concentrations of 2,4-D, NAA and Dicamba on date palm callus (*Phoenix dactylifera* L.) using protein profile and RAPD markers

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Abstract Genetic stability and uniformity of *in vitro*-derived date palm plants has a major importance to ascertain true-to-typeness of produced plants. The goal of present study was to evaluate the genetic toxicity of different plant growth regulators on date palm callus at initiation stages using protein patterns and RAPD analysis. Date palm offshoots of Hillawii cultivar were dissected, apical meristems were divided into four segments and cultured on callus induction medium containing the plant growth regulators as 2,4-D at 50 and 100 mg/L; NAA at 30 mg/L and Dicamba at 10 mg/L. The changes occurred in protein profile of callus when treated with high concentration of 2,4-D (100 mg/L), including loss of normal fragments (19 and 66 KDa polypeptides in control), as well as, appearance of new fragments, while at low concentration of 2,4-D (50 mg/L) and Dicamba treatment, the protein patterns showed no changes compared to control profile. Similar trends of polymorphisms were obtained with RAPD marker. The high concentration of 2,4-D produced more polymorphic fragments in comparison to control treatment. The DNA profile was identical between 2,4-D at low concentration and control. Dendrograms were generated using similarity indices of protein and RAPD results, and revealed that genetic similarity index was high between 2,4-D treatment at low concentration and control, as separated in one subcluster, followed by Dicamba and NAA, while, the highest genetic distance was obtained between 2,4-D at high concentration and control treatment and separated alone in one cluster.

Abbreviations 2,4-D, 2,4-dichlorohydroxyphenoxy acetic acid; 2iP, isopentyl adenine; Dicamba, 3,6-dichloro-2-methoxybenzoic acid; NAA, naphthaleneacetic acid; GD, genetic distance; GSI, genetic similarity index; KDa, kilodaltons; PGRs, plant growth regulators; RAPD, random amplification polymorphic DNA

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1. Introduction

Date palm (*Phoenix dactylifera* L.) is one of the most important members of the family Arecaceae, a dioecious, perennial, monocotyledonous tree cultivated mainly for its nutritive fruits as well as an ornamental plant [3]. Date palm tree plays a pivotal role in the sustainable agriculture in many countries around the world with a long history of cultivation and utilization in Middle East and north Africa for more than five thousand years [10]. The production and utilization of date fruits are increasing over the time, the production was 5.4 million tons in 2001 and reached more than 7.5 million ton in 2009 [1].

For date palm propagation, different propagation methods have been used including seeds, offshoots and tissue culture method. In Iraq, an extensive program has been followed for propagation of different elite date palm cultivars through tissue culture such as Sherafy, Al-Sayer, Hillawii, Khasab, Um Al-Dihin, Barhi, Kantar, Shwaythee, Breem, Alawaidy and Ashkar [2]. However, multiplication of date palm through tissue culture procedure still faces numerous challenges, one of these concerns is off-type of *in vitro*-derived date palm plants, especially when somatic embryogenesis and callus production are employed. Significant group of researchers identified different growth abnormalities (somaclonal variations) including failure to flower or fruiting, dwarfness, loss of chlorophyll in leaves (albino), crop failure, excessive vegetative growth, leaf malformation, single leaf chlorosis, twisted leaf, twisted inflorescence, necrosis of midrib, abnormal terminal bud and bending of whole plant [27,15,19,8,36].

Somaclonal variations of *in vitro*-derived plants could be induced by several causes, one of these causes is the type and concentration of plant growth regulator, among these growth regulators, 2,4-dichlorphenoxy acetic acid (2,4-D) is widely known as a potential mutagenic of callus cells [47,16]. High concentrations (100–150 mg/L) of 2,4-D and another plant growth regulators were employed in date palm callus induction, especially during the early stages of multiplication, more specifically at callus induction stage [55,14,15,42,57], which raised a considerable concern about getting off types of *in vitro*-derived plants.

Different molecular approaches employed in detection of genetic variations in date palm plants including RFLP, RAPD, AFLP, SSR, ISSR, SNPs, iPBs and protein patterns [59,33,9,32,39,37,4,11].

The present study has been conducted to evaluate the genotoxicity effects of different plant growth regulators on tissue culture derived-date palm callus by using protein profile and RAPD markers.

2. Material and methods

2.1. Plant materials

2–3 year old offshoots of date palm Hillawii cultivar were selected and detached from their mother plants. Offshoots were dissected acropetaly until the shoot tips appeared. Shoot tips of 3 cm (apical meristems with leaf primordia) were excised with immature fiber 2 cm in diameter and then applied into antioxidant solution consists of 150 mg/L citric acid and 100 mg/L ascorbic acid to prevent browning [62]. Explants were sterilized in commercial bleach (sodium hypochlorite) 20% containing one-two drops of Tween-20 as emulsifier for 20 min with vacuum and rinsed three times with sterile distilled water. Subsequently transferred to Petri dishes and all leaf primordia were removed except two pairs surrounding the apical meristems (Fig. 1A–D).

2.2. Initiation stage

The apical meristems were divided longitudinally into four segments and cultured on medium composed of basal salts (MS) [53], Table 1), with additional 3 mg/L 2ip and 3 g/L activated charcoal. 2,4-D was used at the concentrations of 50 and 100 mg/L, while Dicamba was used at the concentrations of 10 mg/L, another trial was performed with the auxin NAA at 30 mg/L. The pH of the medium was adjusted to 5.7 with 0.1 N NaOH, before the addition of agar. Media were dispensed into culture test tube with 25 ml in each, subsequently covered with cotton and aluminum foil. Autoclaving at 121 °C and 1.04 kg/cm^2 for 15 min was followed. All cultures were incubated in a culture room under darkness at 27 ± 2 °C until initiation of callus. Subcultures were performed on the same medium and growth conditions every 4 weeks. The required time to obtain callus was varied according to the type and concentration of growth regulator, approximately, 10 weeks for 2,4-D (50 mg/L) and Dicamba (10 mg/L) treatments, 12 weeks for NAA (30 mg/L) treatments and 17 weeks for 2,4-D at high concentration (100 mg/L).

2.3. Extraction of callus protein and SDS-PAGE electrophoresis

After initiation of callus, 300 mg of date palm callus in each growth regulator treatments was ground in liquid nitrogen and homogenized in 3 ml Tris-HCl buffer (0.1 M, pH 7.5) containing 1 mM phenylmethanesulfonylfluoride (PMSF), at 4 °C. The homogenate was centrifuged at 13 krpm for 30 min [22].

Protein content was measured according to Bradford [24] method using Bradford reagent (containing: 100 mg Coomassie Brilliant Blue R-250 in 50 ml 95% ethanol, 100 ml 85% (w/v) phosphoric acid), a crystalline bovine albumin (5–100 µg protein) was used to establish a standard curve at 595 nm.

The concentration of protein in all examined samples was diluted to 40 µg using sterilized distilled water, protein samples were subjected to discontinuous polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions as described by Leammli [43]. Electrophoretic separation was performed at 4 °C for 7 h, using 15% polyacrylamide gels. Staining with commassie brilliant blue and destaining were performed according to Meyer and Lambert [50].

2.4. RAPD procedure

2.4.1. Extraction of callus gDNA

After the initiation of date palm callus in each growth regulators treatment, the genomic DNA (gDNA) was extracted according to Doyle and Doyle [28] by using CTAB (cetyltrimethyl ammonium bromide) method, briefly as follow:

- 250 mg of initiated callus was (as well as young leaves of Hillawii cv. as control) frozen and ground in liquid nitrogen into a fine powder.
- Powder was then mixed with 700 µl of CTAB extraction buffer containing: 20 mM EDTA, 0.1 M Tris-HCl pH 8.0,

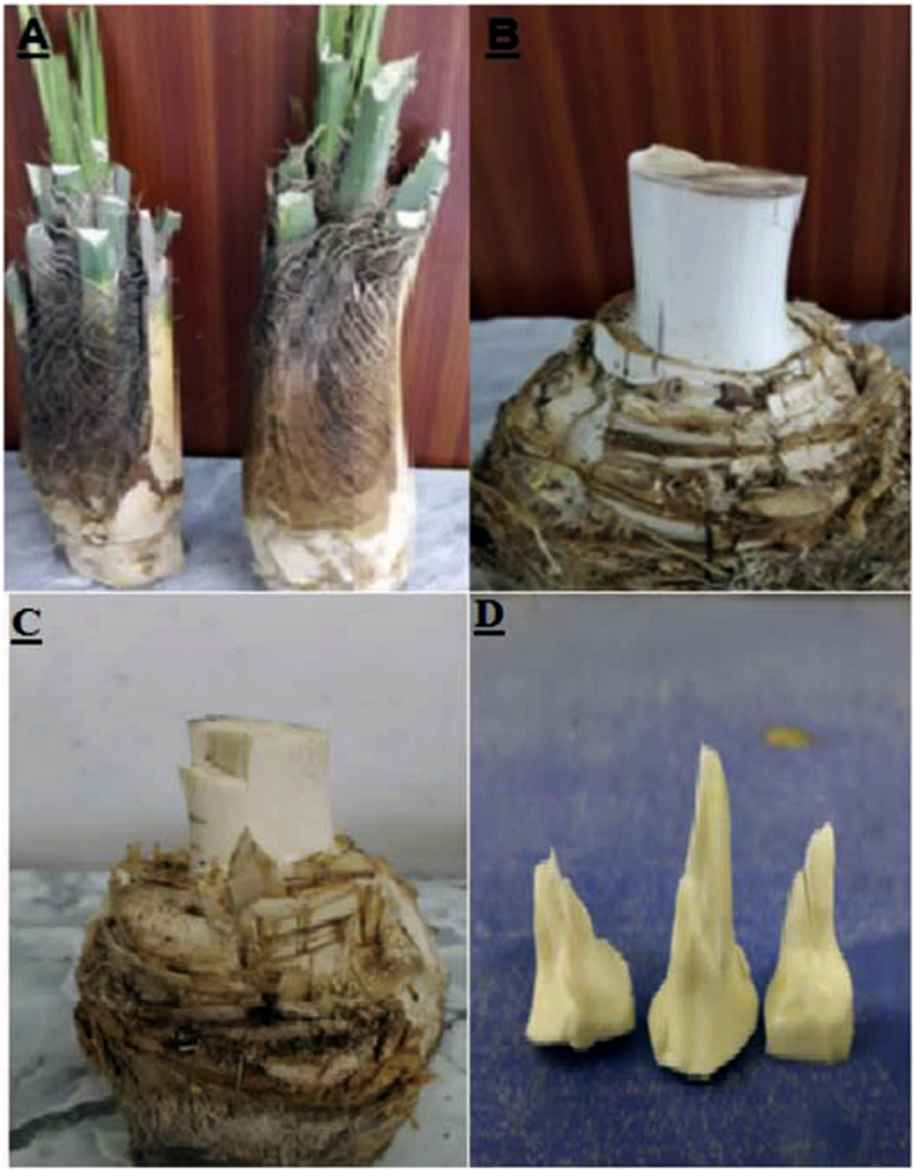

Fig. 1 Date palm offshoot dissecting. A: Date palm Hillawii cv. B: C – Shoot tip before excision. D: Shoot tip after excision.

1.4 M NaCl, 2% CTAB, and 0.4% b-mercaptoethanol added prior to use.

- The solution was then heated up to 65 °C for 45 min, and mixed gently by inversion every 15 min.
- Subsequently, a 500 µl of chloroform-isoamylalcohol (24:1) was added to the mixture and gently mixed for 1 min.
- Centrifugation for 1 min at 12 krpm was followed, and 600 µl as supernatant was transferred into a fresh Eppendorf tube containing 500 µl chloroform-isoamylalcohol (24:1).
- 500 µl of the supernatant was transferred into a fresh tube containing 700 µl ice-cold isopropanol, mixture was mixed very well.
- Another centrifugation for 10 min at 12 krpm was followed, the supernatant was removed, and the DNA pellets were washed with 70% ethanol (700 µl).
- Samples were dried at RT.
- Ressuspension of the pellets was followed with 100 µl TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0); and incubated for one hour at 37 °C.

2.4.2. Quantification of DNA

DNA was quantified in 1.5% agarose gels to check out the gDNA integrity. Electrophoresis was conducted in a 1X TBE buffer [100 mL 10X TBE (0.89 M Tris base, 0.89 M Boric acid, 20 mM EDTA pH 8.0) and 900 mL distilled water] at 60 V for 30 min and then at 120 V for 1.5 h. The gDNA was stained with 0.5 mg/mL of ethidium bromide. gDNA concentration was measured by Nano-Drop spectrophotometer (Bio-Rad, USA) at A260/280, gDNA templates were diluted to 30 ng/µl with TE buffer [3].

2.4.3. RAPD primer description and PCR amplification

Four different Decamer nucleotide primers manufactured by Bioneer-Korea were used for RAPD analysis (Table 2, primer description). Each polymerase chain reaction (PCR) was carried out in a 25 µL volume containing 30 ng template DNA, 1.5 mM $MgCl_2$, 0.32 mM dNTPs, 1X *Taq* DNA polymerase buffer, 10 pmol oligonucleotide primer and 2 units of *Taq*

Table 1 Chemical composition of callus induction medium.

Macronutrient	Concentration mg/L	Micronutrient	Concentration mg/L
KNO_3 1900.00	1900.00	$MnSO_4{\cdot}H_2O$	16.90
NH_4NO_3	1650.00	H_3BO_3	6.20
KH_2PO_4	170.00	$ZnSO_4{\cdot}7H_2O$	8.60
$CaCl_2{\cdot}2H_2O$	440.00	KI	0.83
$MgSO_4{\cdot}7H_2O$	370.00	$FeSO_4{\cdot}7H_2O$	27.84
$NaH_2PO_4{\cdot}H_2O$	170.00	Na_2EDTA	37.25
		$NaMoO_4{\cdot}2H_2O$	25.00
		$CuSO_4{\cdot}5H_2O$	2.50
		$CoCl_2{\cdot}6H_2O$	2.50
Organic components		Concentration mg/L	
Glycine		2.00	
Thiamine		0.50	
Nicotinic acid		0.50	
Sodium dihydrogenorthophosphate		170.00	
Myo-inositol		100.00	
Carbon source		30.00	
Agar		6 g/L	
Activated charcoal		3 g/L	

Table 2 RAPD primer, their sequences, GC %, length and melting temperature.

RAPD code	Sequence (5″–3″)	GC %	Length*	Tm**
OPAR3	GTGAGGCGCA	60	10	32
OPAR8	GTGAATGCGG	70	10	35
P.640	CGTGGGGCCT	80	10	37
P.650	AGTATGCAGC	50	10	32

* Base.
** Melting temperature °C.

DNA polymerase (iNtRon, Biotechnology Inc., Korea). Amplification was performed in a thermal cycler using the following conditions: denaturation at 95 °C for 3 min; 35 cycles of 1 min denaturation at 94 °C, 1 min annealing at 35 °C and 2 min extension at 72 °C; and a final extension at 72 °C for 7 min.

2.5. Protein and RAPD markers data analysis

Protein molecular weight marker of Promega (10–225 KDa, nine fragments) and DNA marker of Thermo Fisher Scientific (100–1500 bp, eleven fragments) were used to detect the precise molecular sizes of each individual fragments using the Photo-Capt MW software 10.0 (Vilber Loumart) and photographed under UV light. Total bands number, polymorphic and monomorphic fragments and fragment lines were scored visually. The following primers parameters were measured as follows (according to Alansari et al. [7]):

$$\text{A – Primer efficiency (\%)} = \frac{\text{the total number of fragment samplified by primer}}{\text{the total number of obtained fragments}} \times 100$$

$$\text{B – Polymorphism (\%)} = \frac{\text{the number of polymorphic fragments}}{\text{the total number of bands amplified by the same primer}} \times 100$$

$$\text{C – Discrimination power (\%)} = \frac{\text{the number of polymorphic fragments amplified by primer}}{\text{the total number of obtained polymorphic fragments}} \times 100$$

All fragments were visually scored as present (1) or absent (0) to create the binary matrix. Only clear and reproducible amplified fragments were considered for estimation the genetic similarity coefficient and distance for all pairs of treatments according to Nie and Li [54], as follows:

$$\text{A – Genetic similarity index} = 2A/(B + C)$$

where (A) number of similar fragments in both treatments, (B) and (C) total number of bands in the first and second treatments.

$$\text{B – Genetic distance} = 1 - \text{GSI}$$

where (GSI) genetic similarity index.

The similarity coefficients were used to construct a dendrogram illustrating genetic relationship using the unweighted pair group mean average (UPGMA) method [60].

3. Results and discussion

3.1. Protein profile analysis

The results of SDS-PAGE electrophoresis analysis of total protein profile from the produced callus of date palm by different growth regulators compared to control treatment (juvenile

Table 3 Protein patterns (presence and absence) in date palm callus produced by different PGRs (mg/L) obtained from SDS-PAGE electrophoresis.

Lane No. of fragment	Molecular weight (KDa)	Plant growth regulators (mg/L)				
		Control	2,4-D 50	2,4-D 100	Dicamba 10	NAA 30
1	19	1*	1	0**	1	1
2	44	0	0	1	0	0
3	66	1	1	0	1	0
4	67	0	0	0	0	1
5	84	0	0	1	0	0
6	100	0	0	1	0	0
Total No. of fragments		2	2	3	2	2

* 1: present of fragment.
** 0: absent of fragment.

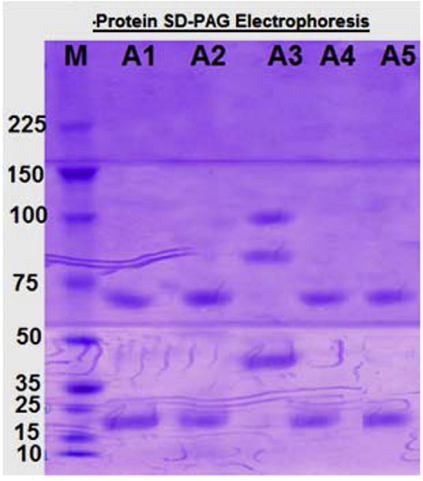

Fig. 2 SDS-PAGE electrophoresis of total soluble protein of date palm callus produced by different PGRs. M: Protein molecular marker (10–225 KDa), A1: Control, A2: 2,4-D 50 mg/L, A3: 2,4-D 100 mg/L, A4: Dicamba 10 mg/L, A5: NAA 30 mg/L.

leaf of Hillawii cv.) revealed no significant differences in protein patterns for 2,4-D at low concentration (50 mg/L) and Dicamba (10 mg/L) compared to control treatment (Table 3, Fig. 2), which produced two fragments with the size of 19 and 66 KDa as molecular weight. The major differences in fragment patterns were distinguished with the increase of 2,4-D concentration to 100 mg/L, where three new expressed polypeptides were observed as 14, 84 and 100 KDa, compared to control treatment. A slight difference was observed in NAA treatment in comparison with control treatment as a new expressed polypeptide was appeared (a size of 67 KDa, Table 3, Fig. 2).

The values of genetic similarities (Table 4) according to Nie and Li index (based on presence/absence of fragments) revealed that the highest GSI values were observed between control treatment and 2,4-D at low concentration (50 mg/L), as well as, Dicamba (10 mg/L) treatments (100%), while, the lowest GSI value was detected between control treatment and 2,4-D at high concentration.

The dendrogram based on genetic distance index was generated for protein profile among different PGRs treatments, and showed a separation of all examined PGRs into two clusters, the first cluster was further separated into two sublclusters, first subcluster included the callus produced by 2,4-D at low concentration, Dicamba and control treatment, while the second subcluster included the treatment of NAA with a genetic distance of 0.5, while the second cluster was included the callus of 2,4-D treatment at high concentration with a highest genetic distance of 1 (Fig. 4A).

Apparently protein electrophoresis is an effective procedure for determination of genetic stability among different treatments, several studies revealed the reliability of this technique in identification of the genetic variations in date palm and many other plants [20,23,61,34,22,48]. Genetic impact of different chemicals has been identified using protein SDS-PAGE electrophoresis including PGRs; hydrogen peroxide and sodium salts [38,35,29,4,51,52].

The present data indicate that the high concentrations of 2,4-D (100 mg/L) led to loss of the polypeptides fragments in control treatment and induce the expression of new polypeptides (44; 84 and 100 KDa), this induction could be an indicator for callus adaptation to high concentration of 2,4-D [22], compared to the identical profile among the treatment of 2,4-D (50 mg/L); Dicamba (10 mg/L) and control.

Table 4 Similarity indices of Nie and Li's coefficients of date palm callus produced by different PGRs (mg/L) obtained from protein analysis.

Treatment	Control	2,4-D 50	2,4-D 100	Dicamba 10	NAA 30
Control	1				
2,4-D 50	1	1			
2,4-D 100	0	0	1		
Dicamba 10	1	1	0	1	
NAA 30	0.500	0.500	0	0.500	1

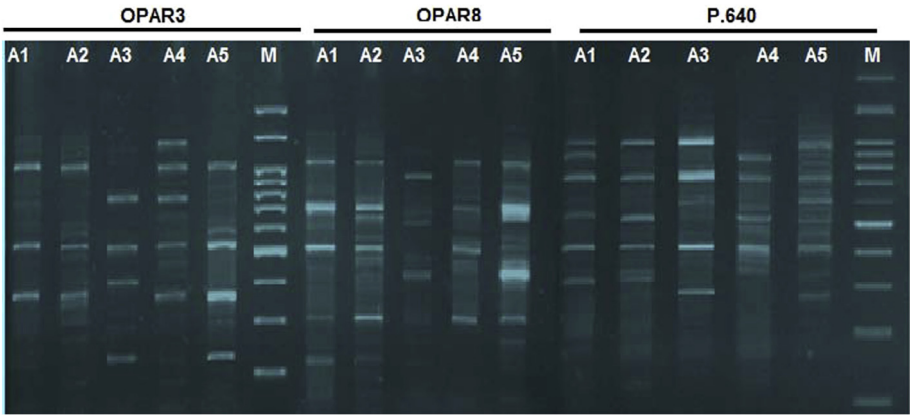

Fig. 3 RAPD marker analysis of date palm callus produced by different PGRs. M: DNA marker (100 bp), A1: Control, A2: 2,4-D 50 mg/L, A3: 2,4-D 100 mg/L, A4: Dicamba 10 mg/L, A5: NAA 30 mg/L.

The adverse effects of 2,4-D at high concentration could be attributed to the genotoxicity which was revealed by many workers on many plants [25,6]. Our results well agreed with the outcomes of previous study which showed the morphological and biochemical adverse effects of 2,4-D at high concentration on date palm callus, including a reduction in fresh; dry weight of callus; total soluble protein; free amino acid content; as well as, increasing of proline content; phenolic compounds and peroxidase activity [13].

3.2. RAPD primer amplification

Using four RAPD different primers, a total of 68 reproducible and clear fragments were generated. Three RAPD primers out of four gave clear results (OPAR3, OPAR8 and P.640). Table 5 represents the results of all primers, 36 fragments of RAPD products were polymorphic. The size of RAPD products was ranging from 112 to 1441 bp, the number of amplified fragments was ranging from 21 to 26 fragments with an average of 22.67 fragments per primer.

Results of RAPD amplification showed that the primer OPAR3 produced 21 fragments with an efficiency of 30.88%, the polymorphism and discrimination percents were 47.60 and 27.78%, respectively. The size of produced fragments was 112–1441 bp, with 10 polymorphic fragments and 21 total amplified fragments. According to RAPD analysis, the results showed that the profile of control and 2,4-D at 50 mg/L treatments was identical, while the increase of 2,4-D to 100 mg/L, the fragment profile showed a significant difference, a new appearance/disappearance of fragments were distinguishable compared to control treatment (Fig. 3). Similar trend of results was scored for both tested primers (OPAR8 and P.640, Fig. 3), where the RAPD profiles proved that the number and size of amplified fragments in control and 2,4-D (50 mg/L) treatments were identical, while the increase of 2,4-D concentration up to 100 mg/L led to a new appearance and disappearance of fragments compared to control profile. Noteworthy, the results of Dicamba treatment were identical to control treatment in OPAR8 primer, while the fragment profile analysis of NAA treatment showed disappearance of two fragments compared to control one in OPAR8 primer.

Additionally, the results showed that the primer OPAR8 produced 12 polymorphic fragments and 21 total amplified fragments, with size of 220–1077 bp, a primer efficiency of 30.88% was observed with this primer, as well as, 33.32% for discrimination power, while, the primer of P.640 produced 14 and 26 polymorphic and total amplified fragments, respectively, with a size range of 285–100 bp and primer efficiency of 38.24% (Table 5).

Similarity indices results (Table 6) of RAPD marker analysis revealed that the highest average of GSI was reported between control treatment and 2,4-D at low concentration which was 90%, followed by control treatment and Dicamba (10 mg/L), while the lowest average of GSI was observed between control treatment and 2,4-D at high concentration (100 mg/L) which was 50%, a percent of similarity index of 71% was detected between control treatment and NAA treatment.

The UPGMA dendrogram of date palm callus produced by different PGRs was computed (Fig. 4B), the dendrogram indicated that the control treatment and 2,4-D at low concentration (50 mg/L) form one subcluster with the lowest genetic distance, while the callus produced by high concentration of 2,4-D was separated in one subcluster with the highest average of GD.

The results of this experiments showed a clear and high productivity of RAPD technique for the analysis of genetic variations during the early stages of tissue cultures of date palm, herein, three decamer RAPD markers amplified DNA fragments with polymorphic features of date palm callus produced by different PGRs, and was efficient in detection of the genotoxic effects of 2,4-D at high concentrations. This result of 2,4-D genotoxicity was in accordance with the results of Cenkci et al. [25] on bean seedlings and Aksakal et al. [6] on maize plants when they used different decamer primers of RAPD.

The RAPD as a powerful technique which approved in this study is in a good agreement with many studies have proven the reliability and reproducibility of RAPD technique in identification of genetic similarities among different cultivars of date palm [17,56,5,31,49,40,11], as well as, detection of true-to-typeness between *in vitro*-derived date palm plantlets and mother plants [26,44,30,58,21,41,18].

The obtained results of differences in decamer primer efficiencies and polymorphism could be related to their sequence differences which affect their sensitivity and binding activity alongside the date palm genomic DNA [3,11].

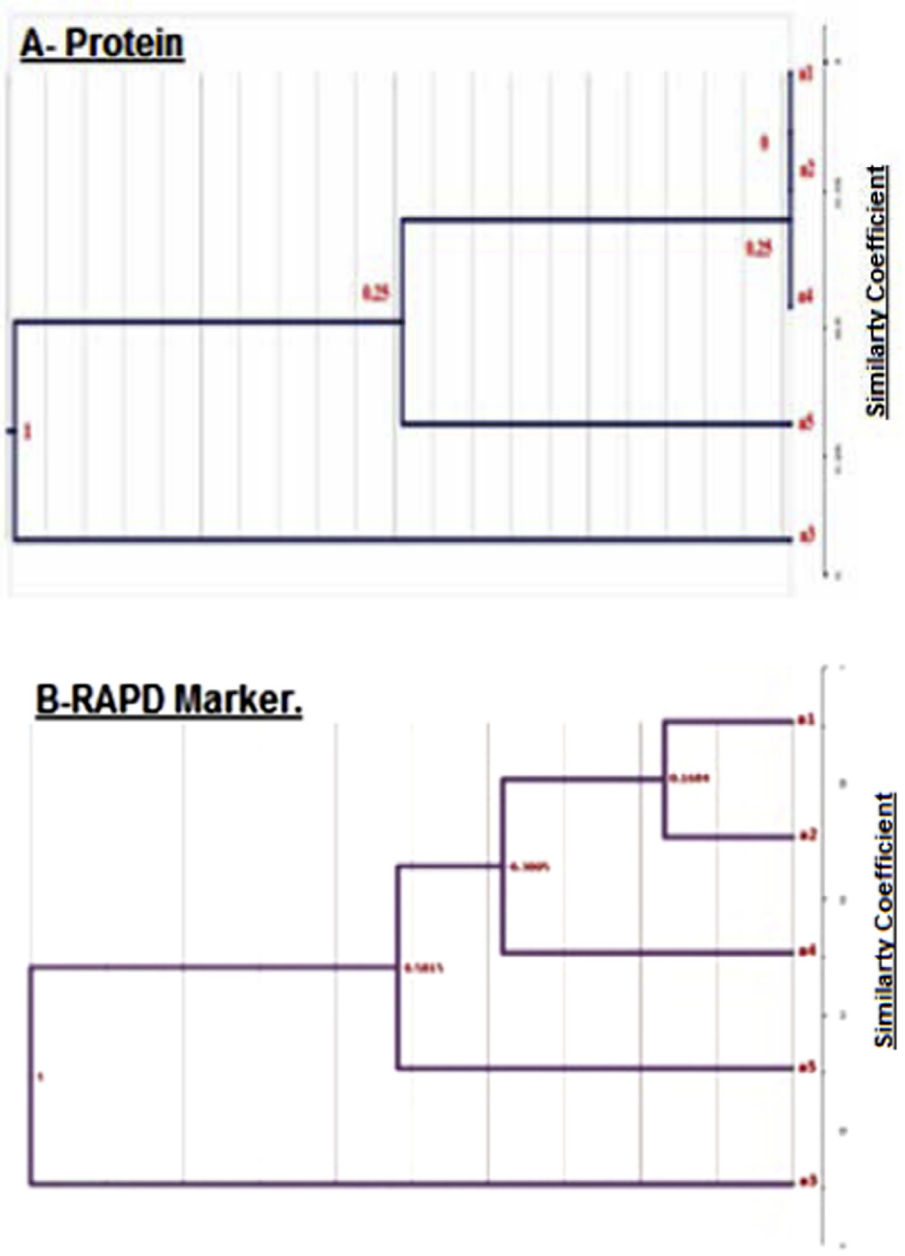

Fig. 4 Dendrograms generated by UPGMA cluster method based on A – Protein SDS-PAGE electrophoresis B – RAPD Markers of date palm callus produced by different PGRs. A1: Control, A2: 2,4-D 50 mg/L, A3: 2,4-D 100 mg/L, A4: Dicamba 10 mg/L, A5: NAA 30 mg/L.

Table 5 RAPD primers with total number of fragments, polymorphic, monomorphic fragments, primer efficiency, polymorphism and discrimination percentages.

RAPD code	Polymorphic fragment	Total No. fragment	Primer efficiency	Polymorphism %	Discrimination %
OPAR3	10	21	30.88	47.60	27.78
OPAR8	12	21	30.88	57.10	33.32
P. 640	14	26	38.20	53.85	39.89
Total	36	68			

Table 6 Similarity indices of Nie and Li's coefficients of date palm callus produced by different PGRs (mg/L) obtained from RAPD markers.

Treatment	Control	2,4-D 50	2,4-D 100	Dicamba 10	NAA 30
Control	1				
2,4-D 50	0.900	1			
2,4-D 100	0.500	0.290	1		
Dicamba 10	0.820	0.730	0.290	1	
NAA 30	0.710	0.660	0.470	0.700	1

In terms of genetic variations induced by high concentration of 2,4-D (100 mg/L) which was obvious by changes in RAPD patterns, including loss of normal amplicons and appearance of new ones compared with control profile, generally, the outcomes of this genetic variations could be caused by several mechanisms, such as the mutagenic activity (deletion or insertion mutation); DNA damage (single or double-strand breaks); chromosomal aberration and modification in genomic template stability [45,25,12,46].

4. Conclusions

Date palm propagation through tissue culture procedure is a preferred way to produce plantlets with a true-to-typeness features. Herein, a confirmation of genetic fidelity was performed at early stages of tissue culture for Hillawii cultivar using protein patterns and RAPD markers. The results revealed that the treatments of 2,4-D at low concentration (50 mg/L) and Dicamba (10 mg/L) showed the highest genetic similarity indices of produced callus to control treatments (juvenile leaves of Hillawii cv.). Additionally, high concentration of 2,4-D (100 mg/L) should be avoided in callus induction medium because of the genetic toxicity of produced callus.

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