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Phytotoxic effect of 2,4-D and dicamba on date palm (*Phoenix dactylifera* L.) tissue cultures at initiation stage

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Abstract. The phytotoxic effect of different concentrations of 2,4-D (2,4-dichlorophenoxyacetic acid) and Dicamba (3,6-dichloro-2-methoxybenzoic acid) on procallus growth of date palm (*Phoenix dactylifera* L.) Hillawii cultivar was investigated. Maximum fresh and dry weights of procallus were obtained in 2,4-D 50 mg/L and dicamba 10 mg/L. A delay in procallus initiation and severe browning responses were observed in cultured tissues at high concentration of 2,4-D (100 mg/L) compared to low ones. A biochemical analysis of date palm procallus revealed that the high concentration of 2,4-D (100 mg/L) led to a significant accumulation of total carbohydrates, free proline content, total phenolic compounds and high activity of peroxidase, as well as, a significant reduction in free amino acid and total soluble protein production, compared to low concentration of 2,4-D 50 mg/L and Dicamba 10 mg/L.
Key Words: Biochemical analysis, auxin, phytotoxicity, micropropagation, physiological responses.

Introduction. Date palm (*Phoenix dactylifera* L.) of the family Arecaceae is one of most important tree crops around the world, especially for Middle East countries, distributed widely throughout North Africa, Middle East, South Sahel areas of East and South Africa, South West USA, Central and South America, as well as, Southern Europe (Al-Shahib & Marshall 2003; Al-Khalifah et al 2012).

Date palm trees play an important role in the life, and their socio-economic importance in many countries in Middle East and South Africa is obvious, their cultivation generates main income source for farmers (El-Hadrami & Al-Khayri 2012). Dates production was approximately 7.4 million tons and worth 3.6 billion US dollars per annum, 69% of the date world production comes from Iraq, Iran, Saudi Arabia, Pakistan and Egypt (FAOSTAT 2011; Abass 2013a).

Date palm is a dioecious plant, which has separate female and male plants, and there are different pathways of date palm propagation, including sexual propagation, which considered the most convenient method, but 50% of the progeny is male, hence, the females produce a small and poor quality dates (Jain 2012). Second propagation method is relay on offshoots which produced from axillary buds during the juvenile life of the palms, with many disadvantages such as, offshoots develop slowly, their numbers are limited and varies from 10-30 depending on cultivar and cultivation practices used (Jain 2012).

Third propagation procedure is micropropagation, which defines as true-to-type propagation of a selected genotypes using *in vitro* technique (Aaouine 2003; Al-Khayri 2005, 2007), large scale multiplication of date palm could be one of the most suitable way to satisfy the world demand of date palm elite cultivars which estimated to be 1-2 million plants per year (Jain 2007).

The vegetation propagation mechanism depends on both induction and multiplication of shoot meristems as potential plants, two potential routs are used commonly for date palm micropropagation, either by direct organogenesis through formation of organs directly from the culture explants, or by indirect organogenesis by

production of mass of unrecognized cells called callus, which by adjusting the ratio of auxin/cytokinin most of callus will differentiate into organs or somatic embryogenesis (Al-Khalifah & Shanavaskhan 2012).

There are numerous obstacles confront date palm micropropagation such as microbial contamination, browning of tissues and media, vitrification, shoot tips necrosis and somaclonal variation (Abass 2013 a,b).

Date palm micropropagation throughout somatic embryogenesis consists of several steps, including culture initiation, callus proliferation, embryogenesis and germination, embryo multiplication, shoot elongation and root development (Al-Khalifah & Shanavaskhan 2012). The initiation stage of date palm micropropagation is normally achieved by employing high concentration of 2,4-D with or without other growth regulators, or with combination of other auxins and cytokinins (Ibraheem et al 2010; Al-Khayri 2011).

High concentrations of auxins were employed in date palm callus induction (Al-Khalifah et al 2010). Table 1 summarize the type and concentrations of auxin as well as the date palm cultivar, briefly, a range of 10-150 mg/L of 2,4-D and 3 mg/L of 2ip were used in callus induction with different cultivars of date palm. Thus, high concentrations of auxins during the early stages of date palm micropropagation, more specifically at callus induction stage alongside with long phase of callus induction could be one of most acceptable explanation for undesirable morphological, physiological as well as genetic abnormalities (Omar & Novak 1990). 2,4-D as a growth regulator is known to cause mutation (Al-Wasel 2000, 2001; Kunert et al 2003; Ramage et al 2004).

Table 1

Auxins and their concentrations for date palm micropropagation

No.	*Growth regulator*	*Conc. mg/L*	*Purpose*	*Cultivar*	*Reference*
1	2,4-D 2ip	100 3	Callus induction	Different cultivars	Tisserat (1983)
2	2,4-D Kinetin BA	10-100	Callus induction	Hillawii	Mater (1988)
3	2,4-D	100	Callus induction	Hillawii	Omar & Novak (1990)
4	2,4-D 2ip	50 3	Callus induction	Zaghlool	Taha et al (2003)
5	2,4-D 2ip	100 3	Callus induction	Khanizi Mordarsing	Eshraghi et al (2005)
6	2,4-D 2ip	100 3	Callus induction	Bartamuda Gondila	El-Din et al (2007)
7	2,4-D 2ip	100 3	Callus induction	Different cv.	Asemota et al (2007)
8	2 4-D 2ip	100 3	Callus induction	Sukary	Alkhateeb (2008)
9	2,4-D 2ip	100 3	Callus induction	Khalas	Kamla Ibrahim & Elmeer (2009)
10	2,4-D 2ip	50 3	Callus induction	Zaghlool	Ibraheem et al (2010)
11	2,4-D	50-200	Callus induction	7 Nigerian cv.	Sani et al (2010)
12	2,4-D 2ip	100 3	Callus induction	Different cultivars	Al-Khayri (2011)
13	2,4-D 2ip	25-100 3	Callus induction	Breem	Khaerallah & Ibraheem (2012)
14	2,4-D 2ip	100 3	Callus induction	5 Pakistani cv.	Abul-Soad (2012)

The objective of present study was to evaluate the phytotoxic effect of different concentrations of 2,4-D and dicamba on date palm tissues at callus initiation stage by analyzing morphological and biochemical parameters.

Material and Method

Plant materials. 2-3 years old offshoots of date palm Hillawii cultivars 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) (Figure 1) 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 (Tisserat 1991). 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. Then they were transferred to Petri dishes and all leaf primordia were removed except two pairs surrounding the apical meristems (Figure 1 A-C).

Figure 1. Date palm offshoot dissection. A-B - Date palm shoot tip before removal form Hillawii cv.; C - Shoot tip after removal; D - Four segments of shoot tips.

Initiation stage. The apical meristems were divided longitudinally into four segments (Figure 1D), and cultured on medium composed of basal salts (MS) (Murashige & Skoog 1962; Table 2), with additional 3 mg/L 2ip and 3 g/L activated charcoal. 2,4-D was used at the concentrations of 1, 50 and 100 mg/L, while dicamba was used at the concentrations of 1, 5 and 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 aluminium 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.

Effect of 2,4-D and dicamba on morphological parameters. All the incubated cultures were observed at growth chamber, and once the procallus emerged on each treated explants, the following morphological parameters were measured:

A. Initial period (day) for callus initiation;

B. Percentage of callus initiation. The percentage of callus initiation was calculated following the formula:

$$\%\ \text{of callus initiation} = \frac{\text{Number of explants produced callus}}{\text{Total number of explants}} x100$$

C. Procallus fresh and dry weight (mg);

D. Browning percentage and intensity. All date palm cultures were observed for browning response, and the indicator of Abul-Soad (2012) was followed as expression of: -, +, ++ and +++ with the interpretation of no response, poor, moderate and high respectively (Figure 2).

Table 2

Chemical composition of used medium (MS) at callus initiation stage

Macronutrient	*Concentration mg/L*	*Micronutrient*	*Concentration mg/L*
KNO_3	1900.00	$MnSO_4.H_2O$	16.90
NH_4NO_3	1650.00	H_3BO_3	6.20
KH_2PO_4	170.00	$ZnSO_4.7H_2O$	8.60
$CaCl_2.2H_2O$	440.00	KI	0.83
$MgSO_4.7H_2O$	370.00	$FeSO_4.7H_2O$	27.84
$NaH_2PO_4.H_2O$	170.00	Na_2EDTA	37.25
		$NaMoO_4.2H_2O$	25.00
		$CuSO_4.5H_2O$	2.50
		$CoCl_2.6H_2O$	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	

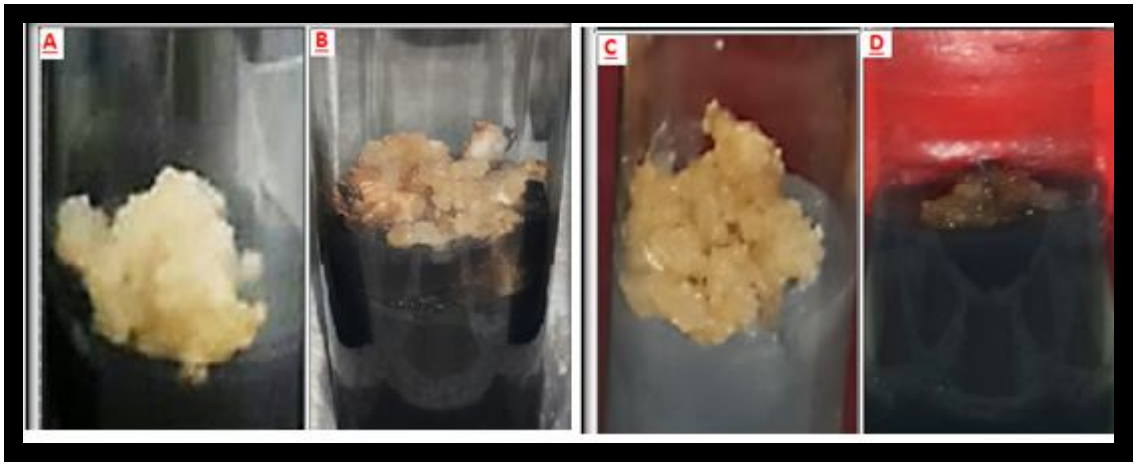

Figure 2. Browning scale according to Abul-Soad (2012). A - no response; B - poor, C – moderate; D - high.

Effect of 2, 4-D and dicamba on biochemical parameters.

Total carbohydrates. The total carbohydrates were quantified as glucose by anthrone technique adopted from Watanabe et al (2000).

Free proline content. Free proline content was measured spectrophotometrically according to Bates et al (1973), by utilizing sulphosalicylic acid and ninhydrin reagent.

Total phenolic content. The total phenolics content was determined with the Folin-Ciocalteu reagent according to the method of Singleton & Rossi (1965). Gallic acid was used as a reference standard; results were expressed as milligram of Gallic acid equivalent (mg GAE/g).

Free amino acids. The free amino acids were determined according to the method of Lee & Takahashi (1966) by utilizing ninhydrine reagent.
Total soluble proteins. Total soluble proteins were quantified using the procedure of Bradford (1976).
Peroxidase activity. Peroxidase activity was detected according to the method of Kim & Yoo (1996). Each one unit of peroxidise catalyzing the oxidation of guaiacol in one minute U/min/g.

Statistical analysis. A complete randomized design was employed in all experiments, the results presented here were analyzed by using the software SPSS for windows (version 10.0). Statistical significant was confirmed by ANOVA (Analysis of variance) and with revised least significant difference (RLSD) test at the probability level of 0.01, with four replicates for each parameter. All results were expressed as mean and standard deviation of the mean

Results and Discussion

Effect of 2,4-D and dicamba on morphological parameters. According to the statistical analysis, all of examined morphological features were significantly affected by the type of auxin, as well as, the utilized concentration (Table 3). Low concentration of 2,4-D (1 mg/L) and dicamba (1 and 5 mg/L) alongside with 2ip (3 g/L) did not stimulate any callus growth during the duration of exposure, shoot tip segments on MS medium supplemented with these low concentrations of auxins did not developed into swelling or procallus over the time of incubation. However, the 2,4-D of concentration 50 mg/L showed an increase in the fresh and dry weight of Hillawii cv procallus and reported the values of 977 and 211 mg, respectively, followed by the treatment of dicamba at 10 mg/L which were 812 and 163 mg, respectively. The 2,4-D at 100 mg/L reported the lowest average of fresh and dry weight of procallus.

Regarding the initiation period (days) to procallus stage, results revealed that the treatments of 2,4-D (50 mg/L) and dicamba (10 mg/L) showed the lowest mean values of required time to induce callus growth which were 67.75 and 66.75 days, respectively, with a significant difference than other treatments, while, the NAA at 30 mg/L reported the value of 85.75 days. Instead, 2,4-D at 100 mg/L caused the highest increase in the time requirement to reach procallus stage which is approximately 2 folds more than 2,4-D (50 mg/L) and dicamba (10 mg/L) treatment. Results of callus initiation percentage elucidated that the treatment of 2,4-D (50 mg/L) was the optimal one than other treatments, and reported the percent of 55% with a significant difference than other treatment, followed by dicamba (10 mg/L) and NAA (30 mg/L) treatments, while, the treatment of 2,4-D at 100 mg/L led to decrease the percent of callus significantly initiation to 32.25%, compared to other treatments.

Browning percentage and intensity of treated date palm cultures showed that the treatment of 2,4-D at 100 mg/L led to a severe browning of most cultures and followed by the highest score of browning intensity (+++) according to the scores of Abul-Soad (2012). NAA (30 mg/L) treatment reported the browning percent of 30% and intensity of (+), while no browning response was observed at the treatment of 2,4-D (50 mg/L).

The effect of different concentrations of 2,4-D, dicamba and NAA was significant on morphological parameters, date palm Hillwaii cv. procallus initial period, callus induction percent, fresh and dry weights were markedly stimulated at 2,4-D 50 mg/L and dicamba 10 mg/L, while no callus induction was observed at low concentrations of 2,4-D 1 mg/L and dicamba 1-5 mg/L. It's noteworthy that 2,4-D at 50 mg/L decreased the required time to induce callus up to 2 folds compared to 2,4-D at 100 mg/L, at the same time, 2,4-D 50 mg/L and dicamba 10 mg/L increased the percentage of explants formed procallus as well as fresh and dry weight.

The majority of recently published researches indicated that the induction of date palm callus and somatic embryogenesis generally required the presence of auxin alone or in combination with cytokinin (Asemota et al 2007; Sani et al 2010; Abul-Soad 2012; Khaerallah & Ibraheem 2012).

Table 3

Morphological analysis of date palm procallus of Hillwii cv. as a response to auxin type and concentration

Growth regulator treatment mg/L	*Initial period (day)*	*% Callus induction*	*Fresh weight (mg)*	*Dry weight (mg)*	*% Browning*	*Browning intensity*
NAA 30 2ip 3	$85.75^{b} \pm 4.34^{*}$	$43.25^{b} \pm 2.50$	$722^{c} \pm 22.16$	$141^{c} \pm 6.65$	30.00^{b}** ± 2.15	+***
2,4-D 50 2ip 3	$67.75^{c} \pm 2.75$	$55.00^{a} \pm 3.74$	$977^{a} \pm 20.60$	$211^{a} \pm 8.50$	$0.00^{d} \pm 0.00$	-
2,4-D 100 2ip 3	$120.00^{a} \pm 3.83$	$32.25^{c} \pm 5.10$	$497^{d} \pm 36.85$	$98^{d} \pm 8.50$	$80.00^{a} \pm 0.00$	+++
DIC 10 2ip 3	$66.75^{c} \pm 2.50$	$42.00^{b} \pm 2.90$	$812^{b} \pm 39.47$	$163^{b} \pm 7.50$	$13.75^{c} \pm 2.75$	+

* - Means of four replicates followed by standard deviation, ** - Means within each column followed by the same letter are not significantly different at $p > 0.05$ level determined by RLSD test, *** - no browning response, + - poor, ++ - moderate, +++ - high.

The maximum fresh and dry weight of date palm procallus at the dicamba 10 mg/L is in a harmony with the results of Omar & Novak (1990), hence, higher rates of growth were obtained for oil palm cultures treated with dicamba (Te-Chato & Hilae 2008). Significant increases of fresh and dry weights of Hillawii procallus undergo the treatment of 2,4-D 50 mg/L is in a good agreement with the results of Khaerallah and Ibraheem (2012).

Regarding the browning percentage and intensity, our results revealed that the 2,4-D 50 mg/L did not stimulate and browning responses, while the lowest percentage of browning and intensity were reported at the treatment of dicamba 10 mg/L. High browning responses (80% and +++) were observed at 2,4-D 100 mg/L. The browning phenomenon of date palm tissue culture is considered as one of the most important obstacles confronts the success of cultures establishments and development, with a remarkable effect on the cultured tissues (Zaid 1984).

Browning of cultured tissues could be attributed to the oxidative browning process of the surface of plant tissues which caused by the oxidation of phenolic compounds resulting the formation of quinones with high reactivity and toxicity to plant tissues (Zaid 1984; El-Shafey et al 1999; Dobranszki & Teixeira de Silva 2010; Abohatem et al 2011).

The physiological toxic effect of 2,4-D at higher concentration (100 mg/L) was profound on date palm cultured tissues, the high percent of browning and intensity were accompanied with a significant decrease in fresh and dry weights of procallus, as well as, a decrease in callus induction percentage compared to the low concentration of 2,4-D (50 mg/L), more callus induction and consequently higher values of procallus fresh and dry weights. Thus, browning responses and growth retarding of cultured tissues was reported in this paper is in accordance with many other researches in many plants including Scot Pine, Apple and Date Palm (Laukkanen et al 2000; Dobranszki & Teixeira da Silva 2010; Abohatem et al 2011; Mustafa et al 2013).

Effect of 2,4-D and dicamba on biochemical parameters. The results of biochemical analysis revealed significant differences among auxin treatments at the protection level of 0.01, total carbohydrates mean values was found to be high (35.40 mg/g) at the treatment of 2,4-D (100 mg/g) which is about 2.5 folds more than what was observed at 2,4-D 50 mg/L (14.40 mg/g) of procallus, while, no significant difference was detected between dicamba and NAA (Figure 3 A). Similar trend of results was found with procallus proline content, which increased significantly from 1.32 µg/g to 3.46 µg/g at 2,4-D 50 and 100 mg/L, respectively, while, no significant difference was seen between dicamba and NAA for proline content (Figure 3 B).

The phenolic compounds were found to be high in procallus of Hillawii cv. at 2,4-D treatment of 100 mg/L which was 1.37 mg GAE/g, and this mean value decreased significantly and reached the lowest level at 2,4-D 50 mg/L and NAA 30 mg/L which were 0.60 and 0.80 mg GAE/g, respectively (Figure 3 C).

Opposite was observed for free amino acid content, 2,4-D 50 mg/L and dicamba 10 mg/L reported the highest mean values of free amino acid which were 1.20 and 1.15 mg/g, respectively, while the lowest mean value was seen at 2,4-D 100 mg/L treatment (Figure 3 D).

Results of total proteins revealed that the auxin of 2,4-D 50 mg/L and dicamba 10 mg/L led to accumulate the highest amount of proteins in date palm procallus which were 0.89 and 0.74 mg/L, respectively, while the lowest mean value of protein was reported at the treatment of 2,4-D 100 mg/L (0.40 mg/L), hence, the value of 0.68 mg/L was reported at the treatment of NAA 30 mg/L (Figure 3 E).

Regarding the enzyme activity of peroxidase, the statistical analysis showed that the treatment of 2,4-D at 100 mg/L induced the highest peroxidase activity which reached 25.45 unit/g/min, and was approximately 2.5 folds more than the activity at 2,4-D 50 mg/L and NAA 30 mg/L treatments, which were 10.58 and 15.10 unit/g/min, respectively, while the activity of peroxidase was 17.75 unit/g/min at dicamba 10 mg/L treatment (Figure 3 F).

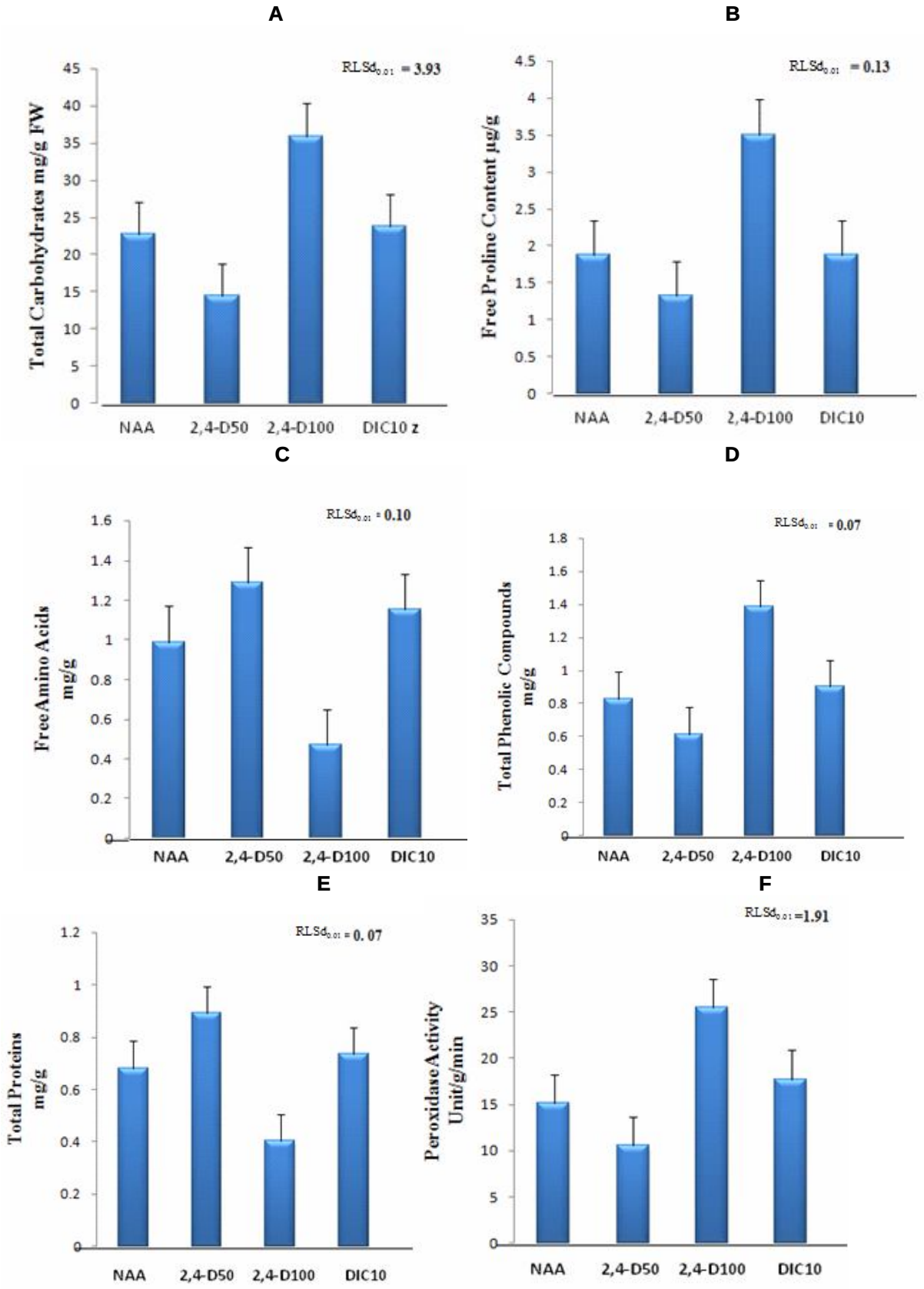

Figure 3. The effect of 2,4-D and dicamba concentrations on some biochemical features of date palm procallus Hillawii cv. A - Total carbohydrates, B - Free proline content, C - Total phenolic compounds, D - Free amino acids, E - Total proteins, F - Peroxidase activity. Error bars indicate the standard deviation, $n=4$.

The chemical analysis of date palm procallus of Hillawii cv. under the effect of tested concentrations of 2,4-D, dicamba and NAA revealed that the average values of total carbohydrates, free proline, phenolic compounds and peroxidase activity were significantly increased at high concentration (100 mg/L) of 2,4-D compared to low concentration ones. Opposite was observed for free amino acids and total soluble proteins which significantly reduced at 2,4-D 100 mg/L.

Regarding total carbohydrates, 2.5 folds increase was observed in 2,4-D 100 mg/L compared to other treatments. Carbohydrates could be an indicator for plant exposed to stressful conditions (Rhodes & Wooltotron 1978; El-Shafey et al 1999).

Free proline content and total phenolic compounds increased significantly at 2,4-D 100 mg/L c up to 3 to 2.5 folds, respectively, compared to other treatments. Many researchers suggest a positive correlation between proline accumulation and plant stress, including low temperature, water defect, salinity, heavy metals exposure and UV radiation (Naidu et al 1991; Hare et al 1998; Munns 2005; Sharma & Dietz 2006). Generally, plant attempt to adjust their proline accumulation to stabilizing sub-cellular structures such as membrane and proteins, scavenging free radicals and buffering cellular redox potential under stress condition (Ashraf & Foolad 2005). High phenolic compounds in date palm cultured tissues at 2,4-D 100 mg/L compared to low concentrations is in a good agreement with many other findings reported that high concentrations of auxins led to accumulate a significant amount of phenolic compounds (Zaid 1984; De Touchet et al 1991; Al-Khateeb et al 2002; Abohatem et al 2011). Peroxidase activity was found to be high at 2,4-D 100 mg/L compared to low concentrations, this finding is in a harmony with the results of Abohatem et al (2011) who reported a positive correlation between peroxidase activity and plant growth regulators.

Both free amino acids and total soluble proteins were found to be high at low concentrations of 2,4-D, dicamba and NAA compared to higher ones, amino acids have generally been considered as precursors to and constituents of proteins and play an important role in plant metabolism and development (Hayat et al 2012).

Looking at all of morphological and biochemical analysis, results showed that the high concentration of 2,4-D 100 mg/L led to increase the browning intensity and retarded the growth of date palm cultured tissues compared to low concentrations of 2,4-D, dicamba and NAA. High carbohydrates values was found to be associated with high production of phenolic compounds, this findings is in accordance with the results of El-Shafey et al (1999), and thus, accompanied with high browning percentage and intensity. High phenolic production is strongly correlated with browning intensity of date palm cultured tissues, many researchers proved this findings in their studies (El-Shafey et al 1999; Al-Khateeb & Ali-Dinar 2002; Mustafa et al 2013; Shehata et al 2014), however, many authors elucidated that high concentrations of 2,4-D led to accumulate a significant amount of phenolic compounds and inhibited the cultures growth (Abohatem et al 2011; Mustafa et al 2013). Additionally, two phenolic compounds were isolated from date palm callus and identified as polar hydroxycinnamic acid (DHC3 and DHC4), their accumulation and relation to browning phenomenon during date palm *in vitro* growth have been proven (El-Bellaj & El-Hadrami 2004).

Our results of reduction in procallus growth at higher concentration of 2,4-D could be attributed to the high pehenolic production, which is in a good agreement with the results of Cvikrova et al (2003) and Shehata et al (2014). High activity of peroxidase in correlation with high phenolic compounds production undergo the high concentration of 2,4-D 100 mg/L is in acordance with the results of Abohatem et al (2011) when they found that the increase in total phenolic compounds in date palm cultures was accompanied with high peroxidase activity, and consequently increase the browning intensity in different cultivars of date palm.

Conclusions. The pronounced decrease of callus fresh and dry weight, the delay of procallus emergence, low percent of callus initiation, as well as, the severe browning response were observed at 2,4-D 100 mg/L treatment, compared with a stimulatory effect of 2,4-D at 50 mg/L and dicamba at 10 mg/L for all of examined features. These

morphological parameters of 2,4-D 100 mg/L treatment were accompanied with a significant increase of total carbohydrates, total phenolic compounds, proline content as well as, high peroxidase activity and decrease of free amino acid and protein content, compared to 2,4-D 50 mg/L and dicamba 10 mg/L treatments.

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