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Influence of Nutrient Medium on Antioxidants Production of Date Palm (*Phoenix dactylifera* L.) Cultivars *in vitro*

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Abstract: Date palm (*Phoenix dactylifera* L.) is the major fruit crop in the Kingdom of Saudi Arabia. Presently, date palm is facing problem in the production of healthy *in vitro* callus tissues. The main objective of this study was to determine the influence of nutrient medium on antioxidants production of callus tissues from date palm cultivars *in vitro*. Four strengths of Murashige and Skoog (MS) culture medium (full, half, one quarter and three quarter strength) were used in the study. The MS strength significantly affected the vitality of the explants and its browning and ability for callus formation. The analysis of variance the cultivars and medium strength showed that they have a clear significantly effect on callus and browning formation of the explants. The highest total phenolic compounds (mg g^{-1}) expressed as gallic acid equivalents induced per explant were 4.274 and 3.262 in Khalas and Shishi cultivars, respectively. The strength of the culture medium was inversely correlated with the antioxidant activity of plant extracts. In conclusion, for callus tissues from date palm cultivars, quarter strength MS medium offered a compromise between optimum growth *in vitro* and antioxidant phenolic accumulation.

Key words: Medium strength, Murashige and Skoog (MS) culture medium, phenolic compounds, browning, ABTS

INTRODUCTION

Date palm (*Phoenix dactylifera* L.) is semi-salt tolerant and medium to severe drought resistant tree. Moreover, its ability to tolerate adverse environmental conditions allowed its cultivation under the arid climatic conditions of Saudi Arabia. The estimated annual production of dates in Saudi Arabia is 1,008,105 tons occupying an area of 156,023 hectares with 23,742,593 date palm trees (MOA, 2012).

Presently, consumption of fruits and vegetables is considered essential and beneficial for health. Many epidemiological studies have demonstrated that high intake of fruits and vegetables is inversely proportional to morbidity and mortality from several chronic diseases such as cancer, cardiovascular diseases, coronary heart diseases, aging, atherosclerosis, neurodegenerative diseases and inflammation (Christen, 2000; Dillard and German, 2000; Prior and Cao, 2000; Liu *et al*., 2004). Fruits, due to the presence of antioxidant contents, have considerable health benefits. For example: these can protect the human body against cellular oxidation reaction, such as protein denaturation, lipid peroxidation and DNA damage as well as oxidative stress-induced nephrotoxicity (Pincemail *et al*., 2002; Kiefer *et al*., 2004; Scalzo *et al*., 2005; Berger, 2006; Saura-Calixto and Goni, 2006; Salah-Ben *et al*., 2012). Presence of vitamin C and E, carotenoids and some polyphenol compounds in date fruit protect against bad health hazards (Ferguson, 2001; Proteggente *et al*., 2002). Also, the antioxidant and antimutagenic compounds in date fruit extract identify free radicals in the system for different functions (Vayalil, 2002; Al-Farsi *et al*., 2005; Mansouri *et al*., 2005; Allaith, 2008; Awad *et al*., 2011).

Previous investigations reported that some steroids, alkaloids and Terpenes are obtained from tissue culture date palm trees for use in the pharmaceutical industries (Bohm, 1980; Staba, 1980; Taha, 1999). Therefore, the main aim of the present study was to investigate the effect of some precursors on the growth, development and synthetic antioxidant from callus of some date palm cultivars.

MATERIALS AND METHODS

Explant preparation

Plant material: Young offshoots weighing between 5-7 kg and about 50-70 cm in height were selected and

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carefully separated from adult date palm trees (*Phoenix dactylifera* L.). Three date palm cultivars (Khalas, Ruziz, and Shishi) growing in Al-Ahsa Oasis, Saudi Arabia were used as plant materials for study.

Sterilization process: Under sterile condition, the explants were surface sterilized and rinsed four times with sterile distilled water. Then, explants were soaked in 3.2% w/v NaOCl (sodium hypochlorite solution) with 60% v/v Clorox a commercial bleach containing 3 drops of Tween-20 per 100 mL for 20 min. The treated explants were rinsed three times with sterile distilled water (Tisserat, 1984; Abo-El-Nil, 1986; Belal and El-Deeb, 1997). After this, the explants samples were transferred to sterile Petri dishes and cut into pieces of approximately 5×5 mm each. All explants were soaked in 70% Ethanol alcohol solution for three minutes. This was followed by three rinses in sterile water and 1.5 g L^{-1} ($HgCl_2$) mercuric chloride solution for 3-5 min before culturing in laminar flow. Then the samples were rinsed 3 times with sterile distilled water (Belal *et al*., 2004).

Culture medium and conditions

Effect of Murashige and Skoog (MS) medium strength: The culture medium consisted of MS inorganic salts (Murashige and Skoog, 1962) with different salt strengths i.e., ¼ MS, ½ MS, ¾ MS and full MS. This was supplemented with (mg L^{-1}) 100 Myo-Inositol; 80 Adenine Sulfate; 170 $NaH_2PO_4.2H_2O$; 30000 Sucrose; 2000 Agar; 7000 (Agar-agar/Gum agar) (Sigma Chem. Co., St. Louis, MO); Activated Charcoal (AC); 2.5 Thiamine-HCl; 2 Biotin; 100 2,4-D and 5 BA. The pH value was adjusted at 5.7 before adding gerlite. The medium was autoclaved at 1.2 kg cm^{-2} equivalent to 121°C for 20 min. The nutrient media was dispensed into small jars having 25 mL of media.

Culture conditions: The cultures were incubated at 25±2°C under total darkness in growth chamber for 6 months. During storage, fresh media was changed three times at an interval of 8 weeks. Data were recorded at the end of each subculture for all the three cultivars. The parameters measured were number of jars contaminated, survival, browning and callus initiation for the cultivars. The idea was to find out the extent of media effect on callus formation and browning phenomenon induced by phenolic compounds formation.

Methods of antioxidants analysis

Chemicals: The chemicals and reagents used for analyzing antioxidant compounds in dates were gallic acid, catechin, sodium nitrate, sodium carbonate, Folin-Ciocalteu's phenol reagent, ascorbic acid, trichloro acetic acid, sodium nitrite, aluminium chloride, methanol were purchased from Merck (Darmstadt, Germany). The 2, 20-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 2,4,6-tripyridyl-S-triazine (TPTZ) were supplied from (Sigma–Aldrich, USA), $FeCl_3$-$3H_2O$, potassium persulphate, sodium acetate, Trolox (6-hydroxy-2,5,7,8 tetramethylchroman-2-carboxylic acid) and sodium carbonate. These were obtained from Sigma–Aldrich of analytical grade.

Extraction: The callus tissues (3 g) were crushed and dry-blended for 10 min with a blender. Later on, the callus tissues were extracted with 100 mL methanol at room temperature (20°C) for 5 h in an orbital shaker. The extracts were then filtered and centrifuged at 4000 rpm for 10 min. The supernatant was concentrated under reduced pressure at 40°C for 3 h using a rotary evaporator (Heidolph-Laborota, Germany) to obtain the callus tissues methanolic crude extract. The crude extract was kept in dark glass bottles for three days inside the freezer until use.

ABTS assay: Antioxidant Activity (AA) was measured by ABTS improved method as described by Cai *et al*. (2004). The ABTS radical cation ($ABTS^+$) solution was prepared by the reaction of 7 μM ABTS and 2.45 μM potassium per-sulphate after incubation at 23°C in dark for 16 h. The $ABTS^+$ solution was then diluted with 80% ethanol to obtain an absorbance of 0.700±0.005 at 734 nm. Then, the $ABTS^+$ solution (3.9 mL; absorbance of 0.700±0.005) was added to 0.1 mL of the test sample and mixed vigorously. The reaction mixture was allowed to stand at room temperature (23°C) for 6 min and the absorbance was recorded immediately at 734 nm. A standard curve was obtained by using Trolox standard solution at various concentrations (ranging from 0 to 15 μM) in 80% ethanol. The absorbance of the reaction samples was compared with the Trolox standard and the results were expressed in terms of Trolox equivalents.

Total phenolic contents-folin-ciocalteau assay: Total phenolics were determined using Folin-ciocalteau reagents (Singleton and Rossi Jr, 1965). About 40 mL of extract of explants and the gallic acid standard were mixed with 1.8 mL of Folin-ciocalteu reagent (prediluted 10-fold with distilled water) and allowed to stand at room temperature (23°C) for 5 min. Then, 1.2 mL of sodium bicarbonate (7.5%) was added to the mixture and allowed to stand for 60 min at room temperature. Then, the absorbance was measured at 765 nm. The results were expressed as mg Gallic Acid Equivalents (GAE) 100 g^{-1} sample according to Shui and Leong (2006).

Data analysis: Data was statistically analyzed by analysis of variance (ANOVA) for the completely randomized design. The treatment means were compared using Least Significant Difference (LSD) at 5 % level of probability. All computations and statistical analysis was performed using the facility of computer and SAS (2001).

RESULTS AND DISCUSSION

Effect of MS medium strength: The effect of MS medium strength and the oxidant was significant on vitality of explants, its browning and ability for callus formation (Fig. 1-3). However, the variance between medium strength on those parameters (vitality, browning and callus formation) of explants was not significant. The analysis of variance for the cultivars and medium strength showed significant effect on callus initiation and browning of the explants under different MS strength of media.

Data in Fig. 1 shows the specific effect on cultivars and the concentration of medium on parameters expressing antioxidants production of date palm during callus formation stage. The effect of treatment on cultivars for vitality of the explants was highest in Shishi followed by Khalasi and Ruziz in descending order. The treatments (concentration of medium) also affected the callus formation considerably. The highest mean values of phenolic compounds formation indicated bro-phenomenon appearance in Shishi followed by Khalasi and Ruziz in descending order. The data in Fig. 1 shows on the X-axis period in months (as mon) and on the Y-axis the mean number of survived explants (A), number of browning explants (B) and number of explants with callus initiation (C). The abbreviations cv. Kh stands for Khalasi, cv. Ru for cv. Ruziz and Sh for Shishsi date palm cultivars.

There was also significant difference among the different MS media strengths on callus formation for different date palm cultivars during six months *in vitro* (Fig. 2). The highest No. of survived explants was in full MS strength and the lowest was in ¾ MS. Furthermore, the highest mean values for browning were in ¼ MS, then ½ MS and the lowest were in full MS strength. This would mean that production of phenolic compounds decreased in full MS strength. The results also showed the highest mean values of callus formation in explants under ¼ and ½ MS, respectively.

Data in Fig. 3 shows the interaction effect between medium strength and three cultivars of date palm on the production of phenolic compounds at callus formation stage that causes browning of explants in the culture medium. The highest mean No. of survived explants and vitality for the explants were in Shishi, Khalasi and Ruziz, respectively. This was true especially for those explants cultured in ¼ and ½ MS strength. Whereas, the highest callus formation was observed in Shishi followed by khalasi and Ruziz especially for those explants cultured in

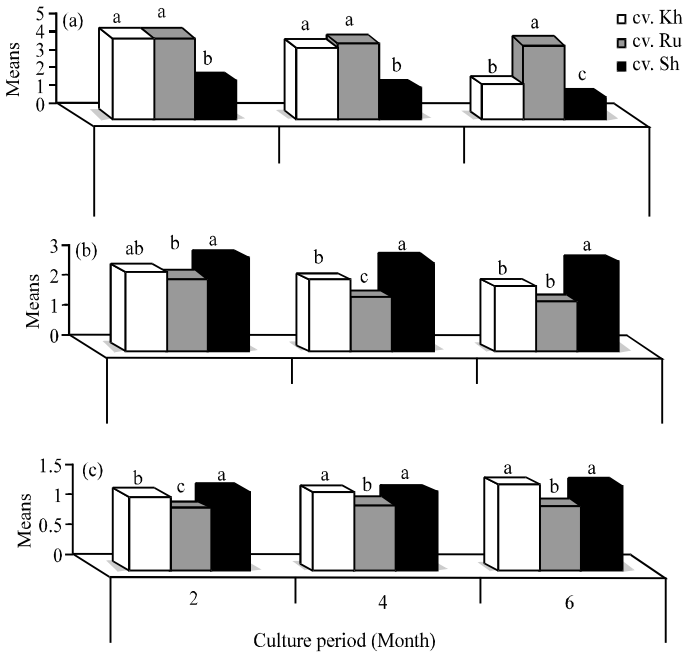

Fig. 1(a-c): Specific effect of cultivars on callus stage of date palm during six months *in vitro*; (a) No. of survived explants, (b) Browning and (c) Callus initiation

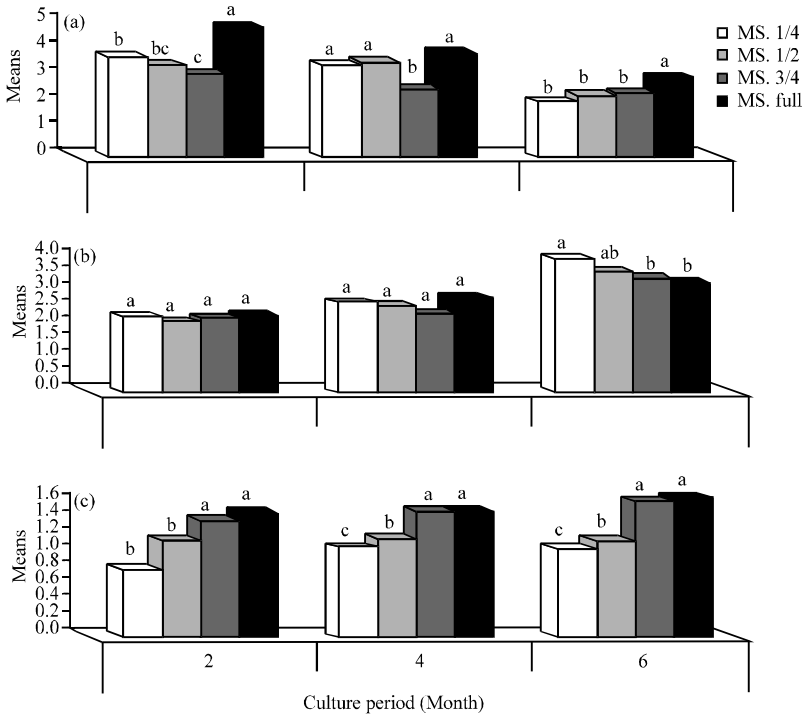

Fig. 2(a-c): Specific effect of different strength of MS medium on callus stage of date palm during six months *in vitro*, (a) No. Of Survived explants, (b) Browning and (c) Callus initiation

full MS strength and the lowest mean numbers for all cultivars were in ¼ and ½ MS strength, respectively.

With respect to browning and production of phenolic compounds (antioxidants), the highest mean values were in Shishi and khalasi with lowest numbers in Ruziz cv. for those explants cultured in ¼ MS and ½ MS strength. However, the explants cultured in ¾ MS medium produced the lowest contents of phenolic compounds that caused browning (Which appeared on the cultivated explants components browning).

It is well known that phenolic components mostly preset in different plant tissues and its concentration differs with the type of plant (explants) and different growing season. The phenolic components are consisted of phenylamine that resemble auxins in its stratum. These include many compounds such as Chlorogenic acid, Phlorglucicol , Orthophenol and Phlorolain etc.

The phenolic components are growth inhibitors due to their action as antiauxins. Many researchers indicated that some phenols protect the natural auxins from deterioration (Co-auxins) and this contradictory effect expands the physiological activity of the phenolic compounds. However, some researchers proved the growth inhibitors role of phenols while some others proved the positive role of some phenols such as phloroglucionol on *in vitro* rooting of some explants like apple (James and Thurbon, 1981; Jones *et al*., 1979).

Regarding date palm, it was proved that phenolic compounds play inhibitory role for the growth of explants. In the presence of oxidizing enzyme like Poly-Phenol Oxydase and Pyroxidaze, some phenol compounds are converted to some poisonous compounds like quinones that cause browning of tissues. This phenomenon is a major physiological problem existing currently which developed interest for the cultivation of date palm from tissue culture.

Generally, browning of explants is attributed to many factors as summarized below.

Factors related to plant itself: These are age of mother plant, type of explants, degree of tissue differentiation and date palm cultivars. Many experiments have proved that degree of browning in shoot tips is less than that found in differentiated leaves (Zaid, 1989a). Whereas, in the cultured flowering primordial (tips) browning degree is less than shoot tips and primary leaves. Further experiments also observed that tissue culture date palm significantly affect the phenolic compounds. However, it was noticed that browning degree in explants cultured in October is less than that cultured during Summer (Al-Kharyi and Al-Marri, 1997).

Factors related to nutrient medium: These are composition of culture medium, concentration and type of

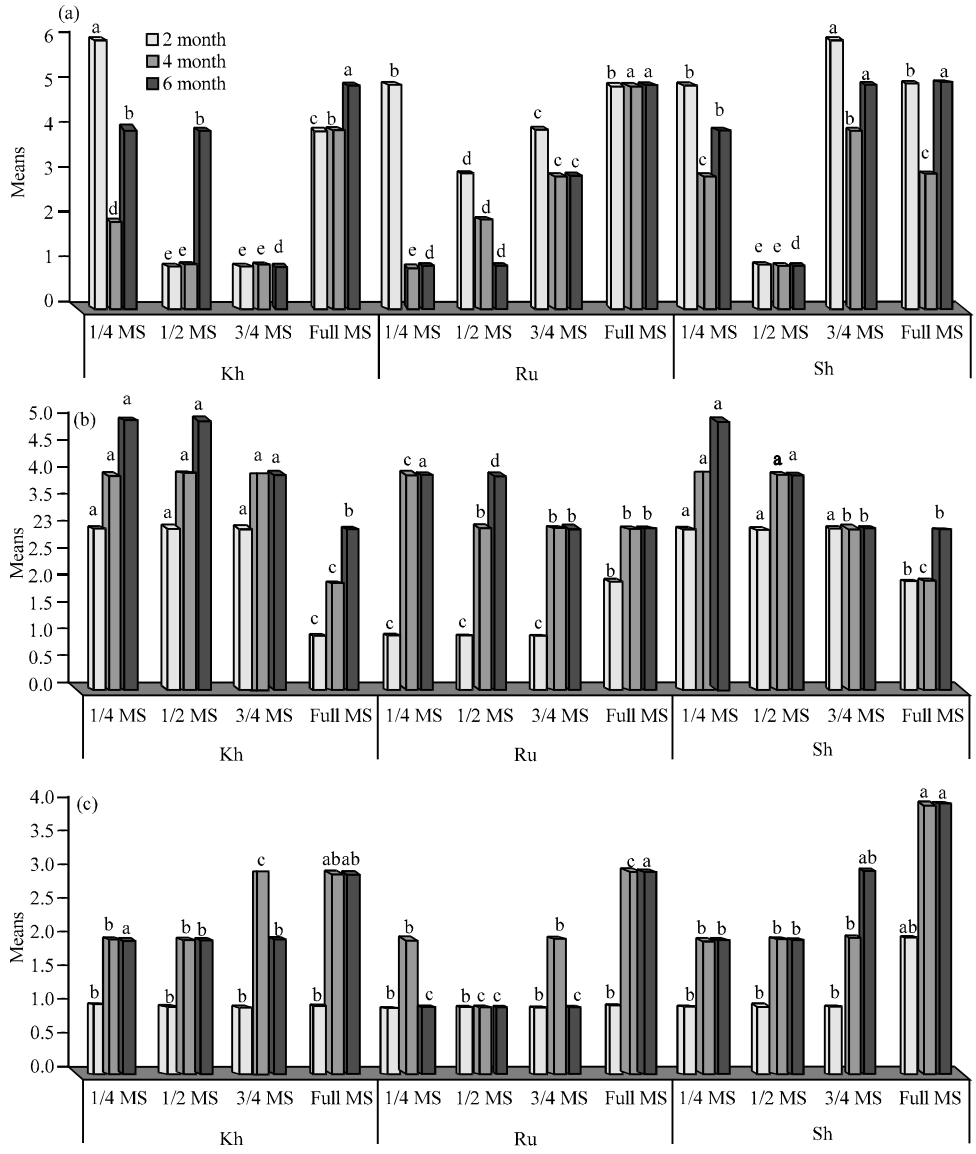

Fig. 3(a-c): Interaction effect of three cultivars date palm and different strength of MS medium on callus stage during six months *in vitro*; (a) No. Of Survived explants, (b) Browning and (c) Callus initiation

growth regulators (hormonal) as well as balance between NH_4^+, NO_3 and K^+. The experiments showed that high concentration of NH_4^+ in the culture medium increases the acidity of the culture medium. This increase in acidity resulted in remarkable decrease in potassium absorption which directly increases the phenolic compounds production and reduces embryonic callus formation (Heller, 1981). This explains the positive results obtained by Sharma *et al.* (1984) concerning limiting browning phenomenon by increasing $KH_2PO_4.2H_2O$ concentration from 170-200 mg L^{-1}. Previously, high concentration of plant hormones like auxins and BAP greatly increased the production of phenolic compounds (Zaid, 1989b; Alkhateeb *et al.*, 2002).

Factors related to atmosphere in culture room: These include temperature and light intensity. Because, some experiments showed that high temperature increases browning and production of poisonous phenolic compounds in the culture medium. While high light intensity increased the browning in explants as compared to darkness (Shehata, 2008; Belal *et al.*, 2008).

Factors related to sterilization and culture technique: For example: Browning increases when high concentration of sterilization agents is used for long time during sterilization process. As there is a concurrent weariness of the explants. Therefore, these should be cultured immediately after sterilization. Because, leaving the

Table 1: Effect of the MS medium strength on total phenolic content and antioxidant production

Cultivars date palm	Strength MS	Total phenolic (mg g^{-1}) as express a gallic acid	Antioxidant activity by ABTS	
			Inhibition (%)	UM trolox
Khalas	¼	4.274[1]±0.003	98.834[1]±0.234	1006.843[1]±4.987
	½	1.235[5]±0.004	46.146[5]±0.248	427.706[5]±2.439
	¾	0.789[8]±0.004	36.571[8]±0.186	324.020[8]±4.113
	Full	0.998[6]±0.02	42.853[6]±0.09	391.654[6]±1.458
Ruziz	¼	0.653[9]±0.002	28.945[9]±0.197	233.401[9]±3.001
	½	0.602[10]±0.08	23.395[10]±0.240	174.934[10]±1.525
	¾	0.595[10]±0.01	23.231[10]±0.02	176.321[10]±2.899
	Full	0.407[11]±0.005	17.915[11]±0.175	117.704[11]±3.535
Shishi	¼	3.262[2]±0.02	97.325[2]±0.555	987.478[2]±2.423
	½	1.691[3]±0.02	67.560[3]±0.103	660.518[3]±1.767
	¾	1.523[4]±0.004	64.149[4]±0.261	624.383[4]±4.802
	Full	0.955[7]±0.01	39.324[7]±0.287	359.867[7]±5.173
LSD at 0.05		1.754	0.404	4.825

explants for a while, before culture, increases the production of phenolic compounds. Furthermore, damages to the explants should be avoided as much as possible during sub-culture stage as the cutting increases the browning phenomenon (Belal and El-Deeb, 1997; Belal *et al*., 2004).

Methods to overcome browning phenomenon: There are many methods to overcome the occurrence of such phenomenon:

- **Addition of active charcoal to the culture medium:** It absorbs phenolic compounds produced in the culture medium thus protecting the explants from browning. It is also recommended to increase the hormonal concentration because some of them are also absorbed by the charcoal (Tisserat *et al*., 1981; Tisserat, 1979)
- **Use of antioxidants such as a mixture of ascorbic acid and citric acid:** Its use in the culture medium gave positive results by limiting the browning of explants (Rhiss *et al*., 1979), But according to some investigators, it gave negative results for limiting browning when added to the culture medium (Zaid, 1989b). However, this mixture is not recommended in the culture medium as it enhances beat growth and increases the contamination of explants (Al-Marri, 1995; Al-Kharyi and Al-Marri, 1997)
- **Use of other compounds with the same Cato oxidant effect:** Like Polyvinyl Pyrrolidone (PVP) and Caffeine (Beauchesne *et al*., 1986; Rhiss *et al*., 1979). These compounds positively affect in limiting the browning of explants
- According to Zaid (1984), the process of frequent transportation and short periods proved effective in reducing the phenomenon of tanning for explants

Effect of the MS medium strength on total phenolic contents and antioxidant production: The highest mean phenolic contents in regenerated callus tissues were 4.274±0.003 mg g^{-1} in Khalas cultivar extracts cultured in ¼ MS and 3.262±0.02 mg g^{-1} in Shishi cultivar extracts cultured in ¼ MS (Table 1).

The effect of different nutrient media and growth regulators on callus tissues regeneration has been previously investigated (Bhat *et al*., 2002; Shasany *et al*., 1998). The present study is the first of its kind for demonstrating the possible effect of MS strength on the total phenolic contents and antioxidant activity in date palm callus tissues. There was an inverse relationship between medium strength and secondary metabolite accumulation in callus tissues cultures. This might be due to the fact that full nutrient media predominantly promote primary metabolism and cellular growth. But in some cases, it hampered the morphological and biochemical tissue differentiation. This hypothesis has been previously verified for the inhibition of differentiation of melon callus cultures by increased concentrations of soluble carbohydrates and selected macronutrient ions (Kintzios *et al*., 2004).

The ABTS inhibitory activity of adventitious callus tissues decreased by increasing the culture medium strength from ¼-½-¾ to full strength (Table 1). A high positive correlation was found between the total phenolic content and ABTS radical scavenging ($r^2 = 0.99$, $p<0.05$) in the extracts of adventitious callus tissues grown in ¼ MS, ½ MS ¾ MS and full MS strength media. Other researchers have also reported a high positive correlation between free radical-scavenging activity and the total concentration of phenolic compounds in plant (Zheng and Wang, 2001; Wagensteen *et al*., 2004; Al-Turki *et al*., 2010). Tawaha *et al*. (2007) observed a positive correlation between the antioxidant activity and total phenolic content for methanolic extracts of 51 Jordanian plant species, including *P. dactylifera*.

CONCLUSION

The present study indicated that, for date palm callus tissues, quarter (1/4) strength of MS medium offers a compromise between optimum growth *in vitro* and antioxidant phenolic accumulation. The highest mean phenolic contents in regenerated callus tissues were 4.274±0.003 mg g^{-1} in Khalas cultivar extracts cultured in ¼ MS and 3.262±0.02 mg g^{-1} in Shishi cultivar extracts cultured in ¼ MS The variation of medium composition could lead to enhance callus tissues regeneration efficiency on cost effective basis (due to low concentration of medium constituents). At the same time, the phytochemical quality of regenerated plantlets could be increased due to their higher content in phenolic compounds with antioxidant activity.

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