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Effect of Ammonium Nitrate on Antioxidants Production of Date Palm (*Phoenix dactylifera* L.) *in vitro*

[1,3]Wael F. Shehata, [1]Mohammed I. Aldaej, [2]Saleh M. Alturki and [1,4]Hesham S. Ghazzawy
[1]Department of Agricultural Biotechnology,
[2]Department of Arid Land Agricultural, College of Agricultural and Food Sciences,
King Faisal University, P.O. Box 400, Al-Ahsa 31982, Saudi Arabia
[3]Department of Plant Production, Faculty of Environmental Agricultural Science,
El-Arish, North Sinai, Suez Canal University, Arab Republic of Egypt
[4]The Central Laboratory of Date Palm Research and Development,
Agriculture Research Center, Giza, Egypt

Abstract: The aim of this study was to determine the phenolic profile of callus tissues of three date palm (*Phoenix dactylifera* L.) cultivars namely Khalas, Ruiziz and Shishi grown in Al-Ahsa Oasis, Kingdom of Saudi Arabia (KSA). The effect of different concentrations of ammonium nitrate on antioxidants activity and *in vitro* production of phenolic compounds was determined by ABTS assay method and their phenolic contents were estimated using the folin-ciocalten method. The effect of treatment for the contamination of explants was highest in Shishi followed by Ruiziz and Khalasi in descending order. The highest value of survival after 2 months was obtained for Shishi (5.42) followed by Ruiziz (5.00) and Khalasi (3.58). After 4 months, Ruiziz cultivar gave the highest value of survival plants (4.67) followed by Shishi (4.50) and Khalasi (3.42). The callus initiation was higher for Ruiziz followed by Shishi and Khalasi taken during the three experimental periods (2, 4 and 6 months). Full concentration of NH_4NO_3 gave the highest value of contamination (5.11) after 2 months followed by zero (4.89), double (4.78) and half concentration (4.33). The total phenolic contents ranged from 2.115±0.010 to 0.653±0.002 mg g^{-1} Gallic Acid Equivalents (GAE) induced per explant. The phytochemical quality of callus showed increases due to high contents of phenolic compounds with antioxidant activity. Overall, half concentration of NH_4NO_3 proved to be the best rate to achieve and to promote high production of important phenolic compounds.

Key words: ABTS assay, ammonium nitrate (NH_4NO_3), antioxidants, browning, callus formation, phenolic compounds, *Phoenix dactylifera* L.

INTRODUCTION

Callus formation is one of the most important growth stages of date palm for *in vitro* micro-propagation that directs the explants to form undifferentiated parenchymal cells via somatic embryogenesis. These undifferentiated cells are exploited in the formation of secondary products and antioxidant without waiting for the plant to reach its maturity stage (Al-Marri, 1995; Belal *et al*., 2008). Subsequently, not only time and effort of those involved in producing those products can be saved but also helps to save the large area utilized for their production. Also, foreign currency is saved for importing these compounds required by vital pharmaceutical industries for human beings (Alturki *et al*., 2013).

Phenols are well-known as antioxidants and veinotonics which may be determinant in the interest of fruit and vegetable consumption for prevention of chronic degenerative diseases, especially against atherosclerosis and concretization (Scalbert and Williamson, 2000; Kris-Etherton *et al*., 2002; Saafi-Ben Salah *et al*., 2012). Plant tissue cultures are the potential sources to produce some active metabolites such as alkaloids, triterpenes and quinines or polyphenols (Oksman-Caldentey and Inze, 2004). The cell suspension cultures represent the best system of cultivation for producing secondary metabolites because fast growth rates can be achieved. Several biotechnological advances have been developed in tissue culture that improved the secondary metabolite production such as optimization of cultural conditions,

Corresponding Author: Wael F. Shehata, Department of Agricultural Biotechnology, College of Agricultural and Food Sciences, King Faisal University, P.O. Box 400, Al-Ahsa 31982, Saudi Arabia

selection of high producing strains of lines, precursor feeding, elicitation, metabolic engineering and transformed root cultures etc. (Sarin, 2005; Al-Ibresam and Al-Meer, 2008; Sae-Lee *et al*., 2011). However, there are very few reports of using macro element to induce phenolic increment in vitro or medium culture. Moreover, it is well known that inorganic metal compounds are one of the most important nutrients that directly or indirectly affect the growth and development of cells and tissues of the plant in addition to their vital role in the formation of these important compounds such as secondary products and antioxidants in particular. One of these inorganic compounds is ammonium nitrate (NH_4NO_3) that is considered as one of the most important inorganic compounds as nitrogen source that is generally needed for its role in completing plant metabolic processes necessary for secondary products and antioxidants formation. So, ammonium nitrate is added to the nutrient MS medium in a high concentration (1.65 g L^{-1}) which demonstrates the high extent of its effect on growth and development of cells. The extent of different concentrations of NH_4NO_3 affects the explant regarding callus formation and the occurrence of browning phenomenon which can turn the color of the explant from white to brown and then black thus resulting in mortality of tissue (Al-Kharyi and Al-Marri, 1997). Belal and El-Deeb (1997) stated that there are certain concentrations that apparently contribute to phenolic substances secretion and antioxidants formation at an optimum rate as those obtained by the naturally grown cultivars in the field without waiting for the plant to reach the maturity stage to get the same quantity of antioxidant at least after eight years in case of date palm. Therefore, this study was carried to investigate the effect of different concentrations of NH_4NO_3 (i.e., 0, 1/2, 1 and 2) of MS medium on cell suspension cultures of date palm (*Phoenix dactylifera* L.) for *in vitru* production of antioxidants and phenolic compounds (Murashige and Skoog, 1962).

MATERIALS AND METHODS

Preparation of explant

Plant material: Young offshoots of date palm selected for study were carefully separated from the parent date palm trees (*Phoenix dactylifera* L.) from three best date palm cultivars (Khalas, Ruiziz and Shishi) grown in Al-Ahsa Oasis, KSA.

Under sterile conditions, the explants were surface sterilized and rinsed four times with sterile distilled water. Then, the explants were soaked in (3.2% NaOCl) containing 3 drops of Tween-20 per 100 mL for 20 min. This process was followed by three rinses with sterile distilled water (Belal and El-Deeb, 1997). The treated samples of explants were transferred to sterile Petri dishes and cut into pieces of approximately 5x5 mm each. All the explants were soaked in 70% Ethanol alcohol solution for three minutes. Then again rinsed three times in sterile water followed by treatment with 1.5 g L^{-1} mercuric chloride solution ($HgCl_2$) for 3-5 min before culturing in laminar flow and then rinsed 3 times with sterile distilled water (Belal *et al*., 2008).

Culture medium and conditions

Nutrient medium: All the explants were cultured in the basal nutrient medium used throughout this study containing inorganic salts (Murashige and Skoog, 1962). For the preparation of the final medium, 20 mL (1.65 g L^{-1}) of NH_4NO_3 were added to about 1000 mL distilled water by stirring.

Effect of combination of ammonium nitrate (NH_4NO_3): All the explants were transferred to a MS medium modified by different concentrations of NH_4NO_3 (0, 1/2, 1 and 2) supplemented with (mg L^{-1}): 100 Myo-Inositol; 80 Adenine Sulfate; 170 $NaH_2PO_4.2H_2O$; 30000 Sucrose; 2000 Activated charcoal; 7000 Agar; 100 2,4-D and 5 BA (Sigma Chem. Co.). The pH of the medium was adjusted at 5.7±0.1 before adding agar and autoclaving the medium at 1.2 kg cm^{-2} equivalents to 121°C for 15 min. The nutrient media was dispensed into small jars of 25 mL of media.

Culture conditions: All the cultures were incubated at 25±2 °C under total darkness in a growth chamber for 6 months. The cultures were kept for a total of 6 months during which they were transferred to fresh media three times at an interval of 8 weeks. Data were recorded at the end of each subculture for the three cultivars. The parameters of this experiment were: total number of contaminated jars, survival of explants and occurrence of browning and callus initiation of the cultivars.

Methods of analysis of antioxidants

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

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

ABTS assay: Antioxidant activity was measured using an improved ABTS method as described by (Cai *et al*., 2004). The ABTS radical cation ($ABTS^{+}$) solution was prepared through the reaction of 7 μM ABTS and 2.45 μM potassium persulphate, after incubation at 23°C in the 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 for 6 min and the absorbance was immediately recorded at 734 nm. A standard curve was obtained by using Trolox standard solution at various concentrations (ranging from 0-15 μM) in 80% ethanol. The absorbance of the reaction samples was compared to that of 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, 1965). The extract of explants (40 mL) or gallic acid standard was mixed with 1.8 mL of Folin-Ciocalteu reagent (pre-diluted 10 fold with distilled water) and allowed to stand at room temperature for 5 min and then 1.2 mL of sodium bicarbonate (7.5%) was add to the mixture. After standing for 60 min at room temperature, the absorbance was measure at 765 nm. The results were expressed as mg Gallic Acid Equivalents (GAE)/100 g sample (Shui and Leong, 2006).

Data analysis: Data was analyzed by analysis of variance (ANOVA) by following the completely randomized design according to Gomez and Gomez (1984). 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 software Inc (SAS, 2005).

RESULTS AND DISCUSSION

Effect of different ammonium nitrate concentrations on callus initiation: The effect of ammonium nitrate (NH_4NO_3) concentration was significant on the vitality of explants, its contamination and survival. However, the variance between NH_4NO_3 concentrations on parameters such as contamination, survival, browning and callus initiation of plants was significant. The analysis of variance for the cultivars and NH_4NO_3 concentrations showed significant effect on different parameters of the explants under different concentrations of medium.

Recently, Alturki *et al*. (2013) stated the importance of MS medium structure containing high nitrogen in the form of (NH_4^+, NO_3^-) in cell division, callus formation and the survival of the plant's vital parameters. The occurrence of any fault either plus or minus in the content of nitrogen in NH_4NO_3 led to the emergence of browning phenomenon by variably emergence of oxidative enzymes and activated significantly leading to discoloration of the plant while it differed in a medium where the best B5 from MS agrees with Abo-El-Nil (1986) who reported that high concentration of glutamine (nitrogen source) in date palm media improved the callus induction and rate of growth.

The important role of MS medium in explant growth and division for callus initiation, in embryogenesis induction and bud formation stages is well documented. This is followed by SH medium to a lower extent than B5 medium and at last ER medium. Also, Mohamed (1996) observed that the diversity of nitrogen sources in the medium greatly helps in callus formation. While, Bekheet and Saker (1998) reported that high concentrations of auxins are required for the initiation of callus formation from the explants that are consistent with the findings of Letouze *et al*. (2000).

Data in Fig. 1a shows the specific effect on cultivars (cvs) and the contamination on different cultivars on parameters expressing antioxidants production of date palm during callus formation stage. Among the different date palm cultivars, the effect of treatment for the contamination of explants was highest in Shishi followed by Ruiziz and Khalasi in descending order as found from the results obtained in 2 months. Also, similar trend was found with the results after 4 months as the following (5.42, 4.92 and 3.33) Shishi, Ruiziz and Khalasi after 6 months. The cultivar Ruiziz gave the highest value (4.92) followed by Shishi (4.58) and Khalasi (1.08). There was also significant difference among the different period (months) and the survival of explants. The highest value of survival after 2 months was obtained for Shishi (5.42)

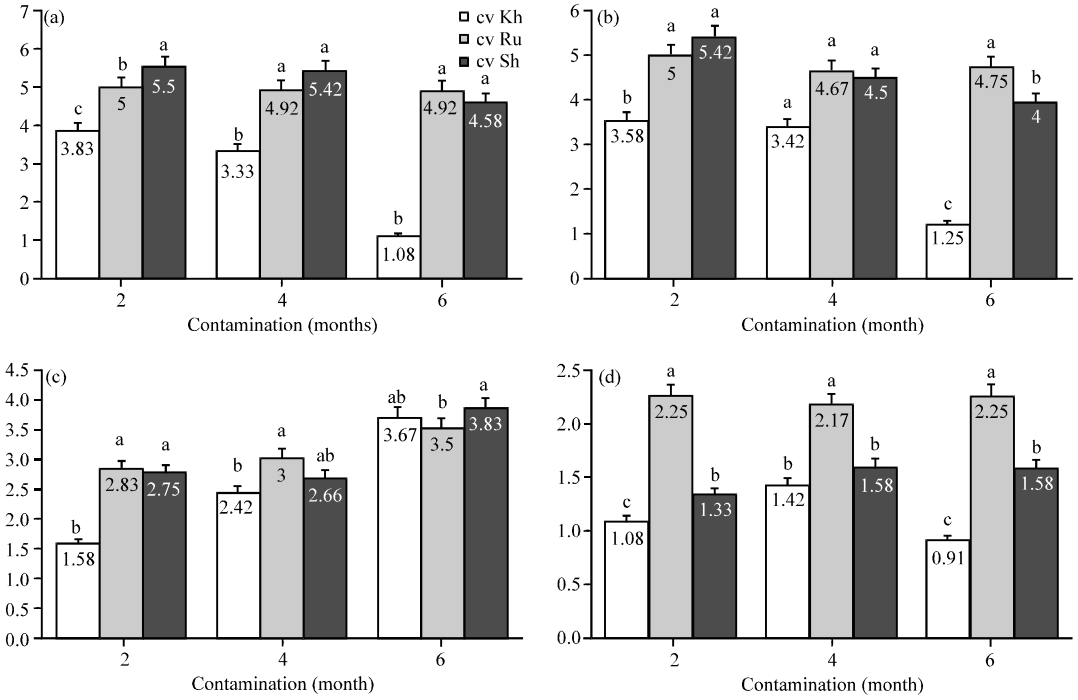

Fig. 1(a-d): Specific effect of three cultivars on callus stage of date palm during six months *in vitro*, (a) Contamination, (b) No. of survived explants, (c) Browning and (d) Callus Initiation

followed by Ruiziz (5.00) and Khalasi (3.58). After 4 months, Ruiziz cultivar gave the highest value of survival plants (4.67) followed by Shishi (4.50) and Khalasi (3.42). A similar trend was observed after 6 moths for the survival of plants which was 4.75, 4.00 and 1.25 for Ruiziz, Shishi and Khalasi cultivars, respectively as presented in Fig. 1b. Data in Fig. 1c shows the specific effect of treatments on the browning of explants for three cultivars. The highest value of browning obtained after 2 months was 2.83 for Ruiziz followed by Shishi (2.75) and Khalasi (1.58). The same trend was obtained after 4 months (3.00, 2.66 and 2.42), but after 6 months, the survival rate of Shishi was superior with a value of 3.83 followed by Khalasi (3.67) and Ruiziz (3.50). Data in Fig. 1d shows the specific effect of three cvs on callus initiation. The clear trend for the callus initiation was superior for the Ruiziz followed by Shishi and Khalasi taken during the three experimental periods (2, 4 and 6 months). The browning phenomenon of cultured tissues caused by the physiological changes within the cultured tissues lead to gradual browning and eventual mortality of the tissues. The browning appears due to the oxidation of phenols within the tissues as reported by Alkhateeb and Ali-Dinar (2002). Moreover, is an established fact that browning of the tissue is correlated with excessive accumulation of phenolics as observed by many researchers (Dubravina *et al*., 2005; Al-Turki *et al*., 2010). While, Tisserat (1979) found that addition of activated charcoal to the nutrient medium inhibited browning of the explant. Similar findings were recorded by Sharma *et al*. (1984) while Anagnostakis (1974) reported contradictory results to the present study findings.

Figure 2a shows the specific effect of different concentrations of ammonium nitrate on callus initiation stage of date palm during different periods (2, 4 and 6 months) on contamination. It was noticed that the full concentration of NH_4NO_3 gave the highest value of contamination (5.11) after 2 months followed by zero, double and half concentration (4.89, 4.78 and 4.33, respectively) and the same trend was found after 4 months. But the results obtained after 6 months showed that the control (zero) concentration) gave the highest value (4.44) than double (3.44) followed by either half or full concentration of NH_4NO_3 (Nadeem *et al*., 2011).

Data in Fig. 2b indicates that zero concentration of NH_4NO_3 gave the highest value in all the study periods (2, 4 and 6 months) and also the same trend was found by the double and full concentration in all treatments. Data in Fig. 2c shows that the value of browning was highest (2.67 and 2.89) after 2 and 4 months, but after 6 months, the full concentration of NH_4NO_3 was superior than half and zero concentration. However, the data clearly showed that half concentration (3.89) after 6 months was the highest than full concentration (3.22).

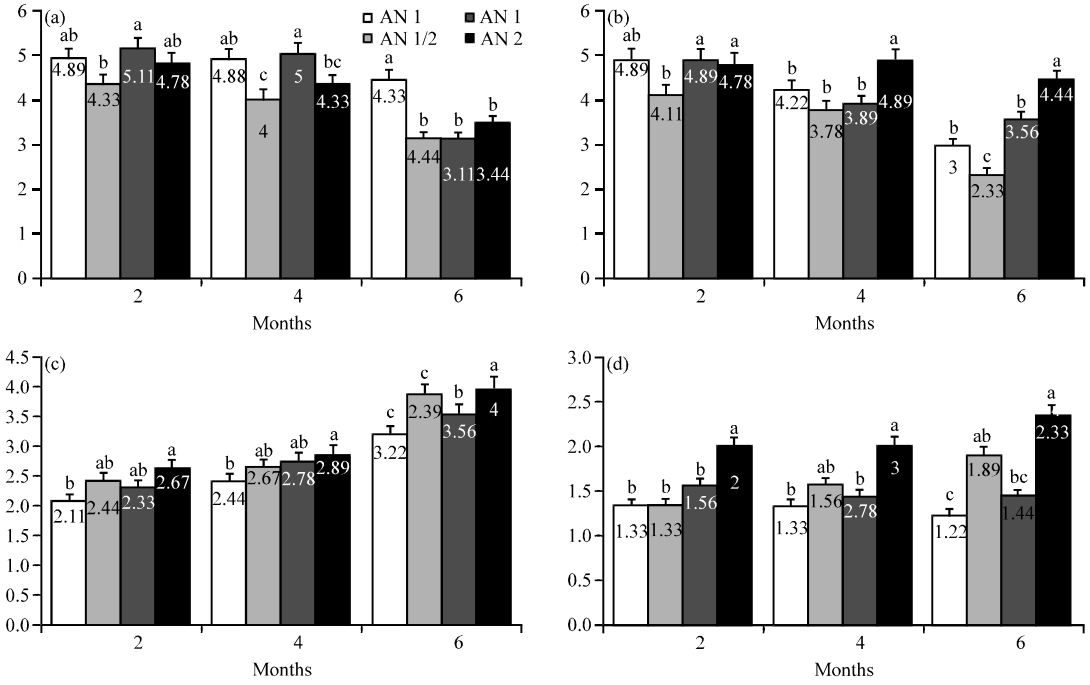

Fig. 2(a-d): Specific effect of different strengths of ammonium nitrate on callus stage of date palm during six months *in vitro* (a) Contamination, (b) No. of survived explants, (c) Browning and (d) Callus Initiation

The callus initiation showed the same trend with double concentration of NH_4NO_3 in all the study periods but the same value was found with half and zero concentration of NH_4NO_3 (1.33) after 2 months. On the other hand, half concentration was superior than full and zero concentration as shown in Fig. 2d as supported by the findings of Asemota *et al.* (2007) who stated the effect of different growth regulators, sucrose and nitrogen on date palm explants cultured on Eeuwen's basal medium. They found that KNO_3 was essential as a source of nitrogen for callogenesis and optimum callus formation at 50 μM.

The results showed the importance of using nitrogen with its optimum concentration (full NH_4NO_3) in addition to keeping a balance between auxins and cytokinins for callus formation and characterization of the cultivars under study. Because, both nitrogen sources (represented in ammonium nitrate) and growth regulators play a cataleptic role in cells growth and division induction as a result of enzymes catalysis specially oxidation-reduction enzymes (respiration enzymes) that helps in energy production (ATP) required for division and growth of cells and formation of phenolic compounds and antioxidants (Al-Marri, 1995; Alturki *et al.*, 2013).

These results are consistent with those of Tisserat (1979) and Mohamed *et al.* (2001) who reported that the type and concentration of auxin play an important role in callus formation and agree with many researchers (Bhargava *et al.*, 2003; Eshraghi *et al.*, 2005) who pointed out that different cultivars differ in callus initiation, browning occurrence, phenolic compounds and antioxidants.

No difference was found between double and full concentration of NH_4NO_3 during the study period (2, 4 and 6 months) on contamination of explants. With respect to Khalas cv, the results indicated that half concentration of NH_4NO_3 gave the highest value of contamination during 2, 4 and 6 months (5.00) with Ruiziz cv followed by half concentration of NH_4NO_3. Similar trend was found with Shishi cv which recorded a value of 6 during 2 and 4 months treatments followed by a value of 5 for 6 months as shown in Fig. 3a. In this context, it was observed that major mineral elements and the essential trace elements are very important in biological processes and play a vital role in normal growth and development and were involved in the prevention of some chronic diseases (Gorinstein *et al.*, 2001; Henriquez *et al.*, 2010).

The interaction effect between cvs and NH_4NO_3 indicated that half and full concentration of NH_4NO_3 gave the highest value of survived plants for Khalasi cv while the Ruiziz cv gave the highest value using the zero and half concentration of NH_4NO_3 in all the treatments during the whole study period (Fig. 3b). A similar trend was found with Shishi cv and the highest value was found

with zero concentration of NH_4NO_3 after 2 months with a value of 6 followed by 4 and 6 months period (5.00), respectively. It is clear from data in Fig. 3c that there is no clear trend for browning between the interaction effect of cvs and NH_4NO_3. On the other hand, Fig. 3d shows that full and double concentration of NH_4NO_3 gave the highest value during the whole study period in all the treatments for Khalas cv and zero concentration for both the Ruiziz and Shishi cvs gave the same trend in all the treatments(Bendini *et al*., 2006; Dlugosz *et al*., 2006; Wojdylo *et al*., 2007).

Effect of the ammonium nitrate strength on total phenolic contents and antioxidants production: Data in Table 1 shows the highest mean for phenolic contents in regenerated callus tissues as 2.115a±0.010 mg g^{-1} in Shishi cv extracts cultured in ½ NH_4NO_3 and 1.955b±0.006 and 1.716c±0.005 mg g^{-1} in Ruiziz and Khalas cvs with 1/2

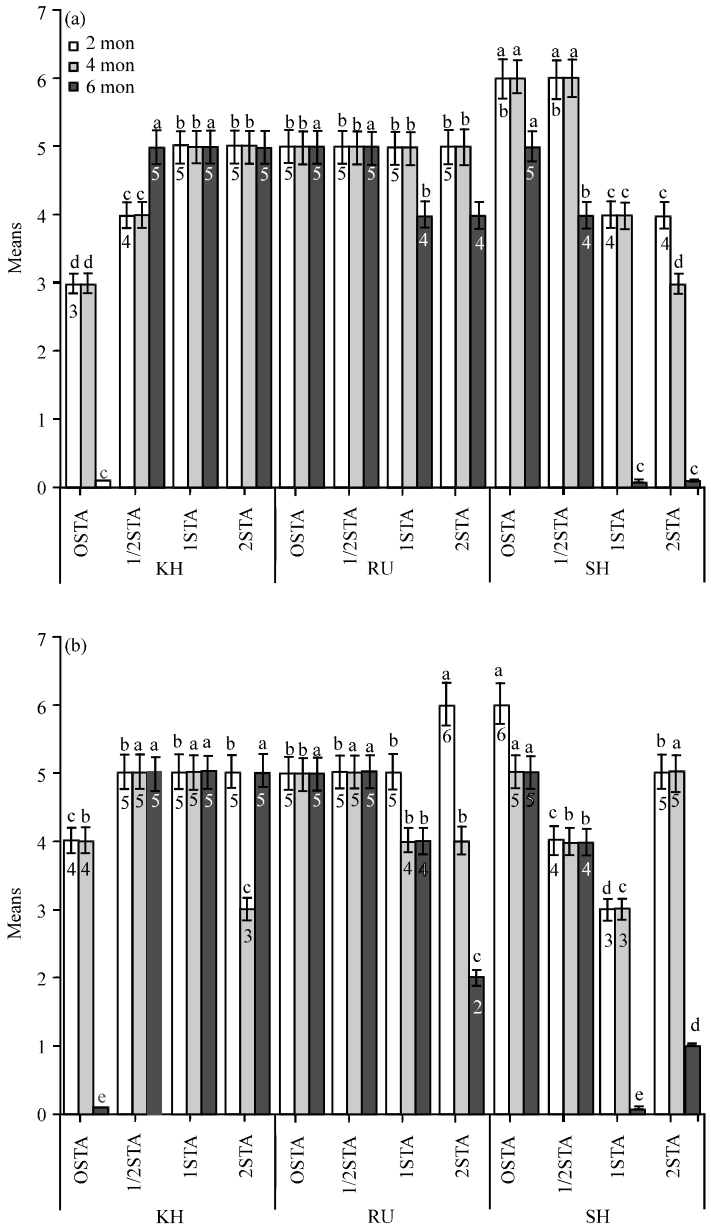

Fig. 3(a-d): Continue

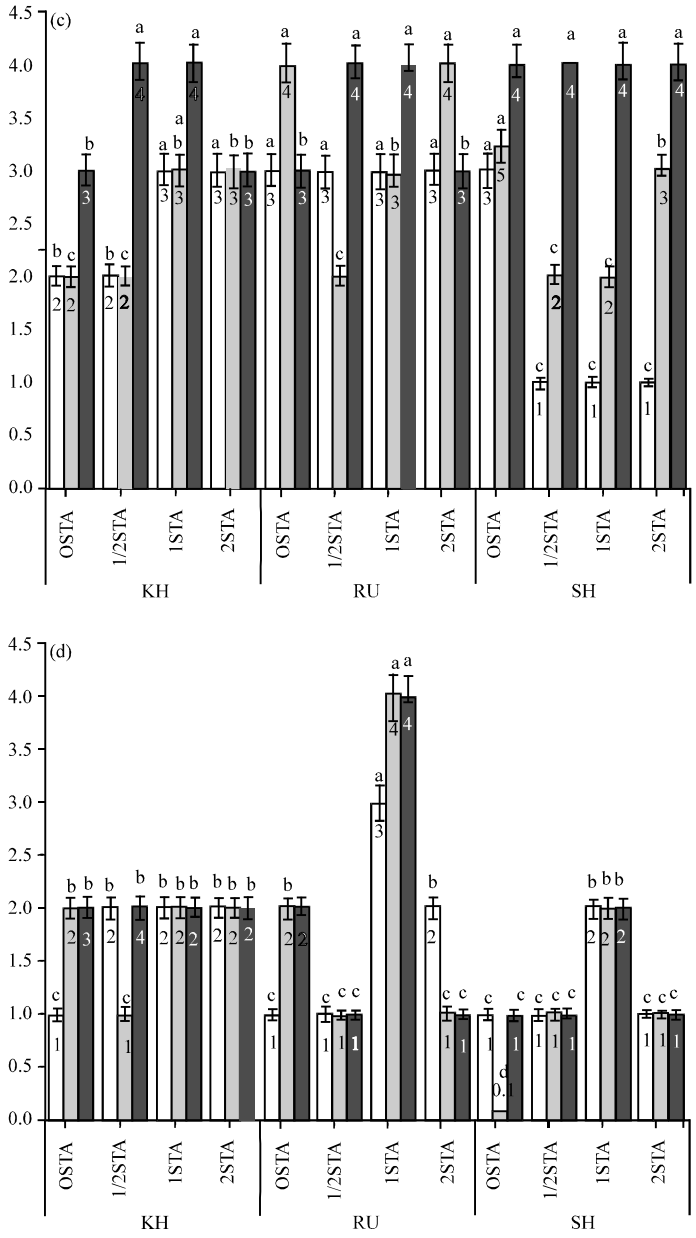

Fig. 3(a-d): Interaction effect of three cultivars date palm and different strength of ammonium nitrate on callus stage during six mons *in vitro* (a) Contamination, (b) No. of survived explants, (c) Browning and (d) Callus Initiation

concentration of NH_4NO_3, respectively. The ABTS inhibitory activity of adventitious callus tissues reached the highest value with half concentration of NH_4NO_3 and then decreased gradually in zero, full and double concentration, respectively. A high positive correlation was found between the total phenolic content and ABTS radical scavenging in the extracts of adventitious callus tissues grown in 1/4, 1/2, 1 and 2 concentrations of NH_4NO_3. Other researchers have also reported a high positive correlation between free radical scavenging activity and the total concentration of phenolic compounds in plant including date palm explants (Zheng and Wang, 2001; Wangensteen *et al*., 2004; Tawaha *et al*., 2007; Al-Turki *et al*., 2010; Alturki *et al*., 2013).

A relatively similar graduation was previously observed for other explants (Lee *et al*., 2010; Sae-Lee *et al*., 2011). Nitrogen sources are important for

Table 1: Effect of the ammonium nitrate (NH_4NO_3) Strength on Total Phenolic Content and antioxidant production

			Antioxidant activity by ABTS	
Cultivars date palm	Strength NH_4NO_3	Total Phenolic (mg g^{-1}m) as express a gallic acid	Inhibition (%)	UM Trolox
	Zero	1.321±0.003^{d}	56.037±0.144^{e}	535.626±2.023^{e}
Khalas	Half	1.716±0.005^{c}	68.290±0.154^{c}	668.669±1.781^{c}
	Full	0.998±0.020^{h}	42.853±0.090^{h}	391.654±1.458^{h}
	Double	1.212±0.008^{g}	47.173±0.168^{g}	437.898±2.435^{g}
	Zero	0.947±0.004^{i}	35.476±0.080^{j}	307.960±1.322^{k}
Ruiziz	Half	1.955±0.006^{b}	75.250±0.063^{b}	743.175±2.332^{b}
	Full	0.653±0.002^{l}	28.945±0.197^{l}	233.401±3.001^{l}
	Double	0.812±0.005^{k}	34.237±0.090^{k}	370.788±3.269^{i}
	Zero	1.330±0.030^{d}	59.468±0.453^{d}	576.743±4.444^{d}
Shishi	Half	2.115±0.010^{a}	78.536±0.278^{a}	782.845±1.663^{a}
	Full	0.955±0.010^{j}	39.324±0.287^{i}	359.867±5.173^{j}
	Double	1.261±0.004^{e,f}	54.473±0.000^{f}	517.273±1.937^{f}
LSD at 0.05		1.754	0.404	4.825

secondary product synthesis of compounds such as alkaloids (Zhong, 2001), anthocyanin's and shikonin from cell suspension cultures (Dong and Chang, 1990). Furthermore, the half concentration of NH_4NO_3 gave the maximum value of phenolic contents and showed the highest browning of callus tissues for all the cultivars such as culture medium composition and the concentration of growth regulators (hormonal) 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 resulting in a markable decrease in potassium absorption which directly increases the production of phenolic compounds and reduces the embryonic callus formation (Zaid and Arias, 1999; Al-Ibresam and Al-Meer, 2008) which supports the positive results obtained by Sharma *et al.* (1984) for limiting the browning phenomenon by increasing $KH_2PO_4.2H_2O$ concentration. It is also important to mention that high concentration of plant hormones such as auxins and BAP greatly increase the production of phenolic compounds (Zaid, 1989; Alkhateeb *et al*., 2002).

CONCLUSION

This present study on cultures of cell suspensions of date palm (*Phoenix dactylifera* L.) has shown that secondary metabolites accumulation was influenced by ammonium nitrate. The highest accumulation of phenolic contents and antioxidants activity were recorded in the medium with half concentration of NH_4NO_3. Consequently, the phytochemical quality of regenerated explants could be increased due to the higher contents of phenolic compounds with antioxidant activity.

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