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

[1]Mohammed I. Aldaej, [2]Saleh M. Alturki, [1,3]Wael F. Shehata 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]Central Laboratory of Date Palm Research and Development,
Agriculture Research Center, Giza, Egypt

Abstract: Antioxidants present in dates are necessary for all physiological processes of humans and animals. In Saudi Arabia date palm is a national fruit tree, produces millions of tons of dates for consumption and is considered a major source of antioxidants. The main aim of this study was to determine the role of potassium nitrate (KNO_3) in the formation of antioxidants from explants collected from date palm cultivars in Al-Ahsa Oasis, Kingdom of Saudi Arabia (KSA) and to monitor the extent of its effect on growth and development of cells during callus formation stage via somatic embryogenesis. The results showed that full concentration of KNO_3 was the best for callus formation in general. While, the half concentration of KNO_3 played an important role for stimulating the explants to form phenolic compounds and the browning emergence for all the cultivars under investigation. On the other hand, the chemical analysis for measuring the phenolic compounds in the explants showed that all the explants formed antioxidants but with varying degrees. The highest mean of phenolic contents was found in those explants cultured with the half concentration of KNO_3 for Shishi cv 2.053±0.010a mg g^{-1} and antioxidant activity by ABTS Inhibition and UM Trolox was 80.694±0.439 and 801.575±2.391, respectively.

Key words: ABTS assay, potassium nitrate, antioxidants, inhibition, UM Trolox, browning, callus formation, phenolic compounds, *Phoenix dactylifera* L.

INTRODUCTION

Date palm (*Phoenix dactylifera* L.) is one of the most important fruits not only in Saudi Arabia but also in the Middle East. It is highly appreciated by the people especially in the Arabian Peninsula particularly in Saudi Arabia (Lunde, 1978; Zaid and Arias-Jimenez, 1999). Tissue culture technique for date palm tree propagation is one of the most modern propagation techniques applied to eliminate many problems associated with date palm cultivation and the obstacles of old well known traditional techniques. Also, further experiments and studies on all aspects of date palm such as cells and tissues were difficult to carry on the date palm tree. Because it is a slow growing perennial tree as well as a dioecious monocotyledonous fruit tree which is contrary to those obtained by *in vitro* propagation that are exactly identically true to type and free of diseases (Tisserat, 1979; Al-Khayri, 2001; Abul-Soad *et al*., 2004; Al-Khateeb, 2008; Belal *et al*., 2008).

Antioxidants are important for several aspects of human health as the dates contain the highest contents of antioxidants and that is why the scientific name of date is *Phoenix dactylifera* L. due to their high content of antioxidants namely dactyliferic acid (Al-Redhaiman, 2004a). Dates are characterized by the varying contents of highly important antioxidants with important medical interest such as polyphenols, vitamin A, C, E, riboflavin, anthocyanin, tannins, thiamine, niacin, caffeine acid, beta-D-glucan-3.1, filafinuh dyes, chloro genik acid, trans snamik acid, kerstin and others (Wajih, 1986). Dates also contain essential minerals such as selenium (Se) that acts as an antioxidants which is highly important for human health as proved by recent studies that antioxidants are

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

necessary for all physiological processes of humans and animals (Al-Redhaiman, 2004b). In addition to that, these act as antifungal, antibacterial and antiviruses (Sae-Lee *et al*., 2011) play important role in repairing and renewal of cells and destroying mutant cells thus increasing the immunity (Sarin, 2005; Al-Ibresam and Al-Meer, 2008). In general, more studies were carried out on date palm as an important source of several antioxidants and free radicals scavenging (Alturki *et al*., 2013).

The level of potassium nitrate concentrations affects the explants with respect to callus formation and occurrence of the browning phenomenon which turns the color of explants from white to brown then black eventually leading to death (Al-Kharyi and Al-Marri, 1997). Shehata *et al*. (2014) stated that there are certain concentrations that apparently contribute to phenolic substances secretion and antioxidants formation to an optimum level as those obtained from the date palm cultivars naturally grown in the field without waiting for the plant to reach its maturity stage to get the same quantity of antioxidant at least after eight years in case of newly cultivated date palm trees. This study was planned to investigate the effect of KNO_3 on cell suspension cultures of date palm (*Phoenix dactylifera* L.) for antioxidants and phenolic production *in vitro* by using different concentration of KNO_3 i.e., zero, half, full and double concentration of MS medium (Murashige and Skoog, 1962).

MATERIALS AND METHODS

Preparation of explants

Plant material: Young offshoots were selected and carefully separated from three adult date palm cultivars (Khalas, Ruziz and Shishi) grown in Al-Ahsa oasis, KSA and used as the plant material in this study.

Sterilization process: The explants were surface sterilized and rinsed four times with sterile distilled water. Then, the explants were soaked in 3.2% NaOCl (equal to 60% Clorox) containing 3 drops of Tween-20 per 100 mL for 20 min and followed by three rinses with sterile distilled water. The sterile explants were transferred to sterile Petri dishes and cut into pieces of approximately 5×5 mm each. All the explants were soaked in 70% ethanol alcohol solution for 3 min. Then the treated explants were rinsed three times with sterile water followed by treatment with 1.5 g L^{-1} Mercuric chloride ($HgCl_2$) solution for 3-5 min before culturing in laminar flow, then again rinsed three times with sterile distilled water (Belal *et al*., 2008).

Culture medium and conditions

Nutrient medium: All the explants were cultured on the basal nutrient medium throughout this study containing inorganic salts. For preparation of the final medium, 20 mL (full strength = 1.90 gm L^{-1}) of potassium nitrate (KNO_3) was added to about 1000 mL distilled water while stirring.

Effect of combination of potassium nitrate (KNO_3): All the explants were transferred to MS medium modified by using different KNO_3 concentrations (i.e., 0, ½, 1 and 2 strengths), supplemented with (in mg L^{-1}) 100 MyoInositol; 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 was adjusted to 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 autoclavable culture tubes of 20 mL capacity.

Culture conditions: All the prepared cultures were incubated at 25±2°C under total darkness in a growth chamber for 8 months. These cultures were kept for a total period of 8 months during which these were transferred to fresh media four times at an interval of 8 weeks. Data were recorded at the end of each subculture for the three cultivars. The experimental parameters such as number of culture tubes for survival, browning and callus initiation for the cultivars.

Methods of antioxidants analysis

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, methanol (purchased 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: The callus tissues (3 g) were crushed and dry-blended for 10 min with a blender. Then, the callus tissues were extracted with 100 mL methanol at room temperature (23°C) for 5 h using an orbital shaker. The extracts were 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 methanolic crude extract of the 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 with 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. $ABTS^+$ 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 immediately recorded at 734 nm. A standard curve was prepared using Trolox standard solution at various concentrations (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-folds with distilled water), allowed to stand at room temperature (23°C) for 5 min and then 1.2 mL of sodium bicarbonate (7.5%) solution was add to the mixture. After standing for 60 min at room temperature (23°C), 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 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 the computations and statistical analysis was performed using the facility of computer and SAS (2005).

RESULTS AND DISCUSSION

Effect of different potassium nitrate strength on callus initiation stage: Data in Table 1 show the specific effect between three cultivars (Khalas, Ruziz and Shishi) and the concentration (strength) of potassium nitrate (KNO_3) on the mean survival number of shoot tips of explants. The

Table 1: Specific effect of three cultivars and different concentrations of potassium nitrate (KNO_3) on callus stage of date palm during eight months *in vitro*

		Culture period (month)				
Parameters and factors	Treatments	2	4	6	8	Mean
No. of survived explants						
Cultivars	Kh	2.42^a	3.75^b	4.17^b	4.42^b	3.69
	Ru	3.00^a	3.33^b	4.75^{ab}	4.75^b	3.96
	Sh	3.08^a	4.58^a	5.25^a	5.50^a	4.60
CV mean		2.83	3.89	4.72	4.89	
KNO_3 strength	0	3.00^a	3.67^a	4.44^a	4.78^a	3.97
	½	3.00^a	4.11^a	4.78^a	5.00^a	4.22
	1	2.33^a	3.89^a	4.89^a	4.89^a	4.00
	2	3.00^a	3.89^a	4.78^a	4.89^a	4.14
KNO_3 strength mean		2.83	3.89	4.72	4.89	
Browning initiation						
Cultivars	Kh	2.42^b	2.50^b	2.83^a	3.50^a	2.81
	Ru	1.83^c	2.00^b	2.17^b	2.75^b	2.19
	Sh	2.92^a	2.92^a	3.17^a	3.83^a	3.21
CV mean		2.39	2.47	2.72	3.36	
KNO_3 strength	0	2.22^a	2.44^a	2.78^a	3.22^a	2.67
	½	2.56^a	2.56^a	2.67^a	3.56^a	2.84
	1	2.11^a	2.33^a	2.78^a	3.22^a	2.61
	2	2.56^a	2.67^a	2.67^a	3.44^a	2.84
KNO_3 strength mean		2.36	2.50	2.73	3.36	
Callus formation						
Cultivars	Kh	1.00^a	1.25^a	1.50^a	1.58^{ab}	1.33
	Ru	0.83^a	1.17^b	1.25^b	1.33^b	1.15
	Sh	0.92^a	1.25^a	1.50^a	1.92^a	1.40
CV mean		0.92	1.22	1.42	1.61	
KNO_3 strength	0	0.78^b	0.89^b	1.11^b	1.11^b	0.97
	½	1.00^{ab}	1.00^b	1.11^b	1.44^b	1.14
	1	1.11^a	1.44^{ab}	1.56^a	1.67^{ab}	1.45
	2	0.78^b	1.56^a	1.89^{ab}	2.22^a	1.61
KNO_3 strength mean		0.92	1.22	1.42	1.61	

*p-values ≥0.05 are significant

data revealed that survival number of explants of culture medium surrounded the explants were reported to be ubiquitous for all culture media under investigation. However, the data showed that Shishi cv. recorded the maximum mean survival number (4.60) in 8 month followed by Ruziz and Khalas cvs. as 3.96 and 3.69, respectively. On the other hand, half the concentration (strength) of potassium nitrate gave the highest mean value of 4.22 followed by double, full and zero concentration (strength) of KNO_3 as 4.14, 4.00 and 3.97, respectively.

Regarding the specific effect of potassium nitrate concentration on browning degree, Shishi cv. recorded the highest mean number (3.21) followed by Khalas and Ruziz cvs. (2.81 and 2.19, respectively). The experimental results showed that the specific effect of concentration (strength) of KNO_3 on browning degree of explant of culture medium surrounded the explant is reported to be ubiquitous for all the investigated culture media. However, the culture media supplemented with half and double concentration (strength) of potassium nitrate gave the most significant of browning degree mean (2.84) when compared with other culture media. However, the Shishi cv. recorded the maximum browning degree mean (3.21) while it was different between browning degree means of Khalas and Ruziz cvs. (2.81 and 2.19, respectively) as shown in Table 1.

Among the different cultivars, Shishi cv. gave the superior mean (1.40) followed by Khalas and Ruziz as 1.33 and 1.15, respectively. While, the interaction between culture media with (KNO_3) concentration (strength) and culture period (month) revealed that double concentration (strength) of potassium nitrate was the best for callus production (1.61) followed by full, half and zero concentration (strength) followed by the mean value of callus initiation as 1.45, 1.14 and 0.97, respectively.

The data in Table 2 show the interaction effect among the three cultivars (Khalas, Ruziz and Shishi cvs.) and the concentration (strength) of KNO_3 on mean survival number of shoot tips of explants. The Ruziz cv. Recorded the highest average (4.25) in 2 months while the Shishi cv. showed the best average in 4 months (3.75). While, the Ruziz cv. recorded the best average in 6 and 8 months as 5.25. In this context, main minerals and essential trace elements were found to be very important in biological processes which play a vital role in normal growth and development as well as in the prevention of some chronic diseases (Gorinstein *et al*., 2001; Henriquez *et al*., 2010).

Also, no differences were found among the three cultivars in 2 months (1.00), while, Shishi cv. Showed the highest average in browning initiation in 4, 6 and 8 months as 3.25, 3.00 and 3.00, respectively. The data show that the highest average of 1.50 was recorded in Shishi cv. in 2 months. The same average was recorded in 4 months in Khalas and Shishi cvs. (1.50), the Shishi cv. recorded the highest average of 1.75 in 6 months while the Ruziz cv. recorded the best and highest value of 3.25 in 8 months.

Callus stage is one of the most important growth stages of date palm *in vitro* micropropagation that directs the explants to form undifferentiated parenchymal cells via somatic embryogenesis (Al-Khayri, 2001; Al-Khateeb, 2008). Those undifferentiated cells are exploited in the formation of secondary products and antioxidant without waiting for the plant to reach its maturity stage "approximately eight years in date palm from the date of cultivation" (Al-Marri, 1995). Those undifferentiated cells are exploited in the formation of secondary products and antioxidant without waiting for the date plant to reach its maturity stage. Subsequently, more time can be saved for those involved in the production of those products and can save vast areas utilized in their production in addition to save the hard currency paid for importing those important materials vital to pharmaceutical industries for human beings (Alturki *et al*., 2013; Shehata *et al*., 2014).

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 as well as the growth of plant itself in addition to their vital role in the formation of those important compounds such as secondary products and the antioxidants in particular. However, one of those inorganic compounds is the potassium nitrate which added to the nutrient medium in high concentration (1.90 g L^{-1}) indicating its high effect on growth and development of cells. That is why, one of the main goals of this study was to identify the intensity of effect of this important product on the formation and secretion of phenolic compounds and antioxidants by the explants in one way or another by using different concentration of KNO_3 at the rates of 0, 1/2, 1 and 2 concentrations of MS medium.

Moreover, MS medium is one of the most important and best nutritive media used for tissue culture that achieve reliable and acceptable results with date palm *in vitro*. This media generally consists of seven principal concentrations of stocks representing (macro and micro) minerals, vitamins and amino acids. The media lakes only the addition of carbohydrates and hormones to be completely sufficient with all nutrients needed by the tissue. In general, it is characterized also by its high content of nitrogen and potassium in the form of NH_4NO_3, KNO_3, $KH_2PO_4.2H_2O$ and KI (Belal *et al*., 2008). Despite

Table 2: Interaction effect of three cultivars and different concentrations of potassium nitrate (KNO_3) on callus stage of date palm during eight months *in vitro*

		KNO_3 strength				
Parameters and time (month)	Cultivars	0	½	1	2	KNO_3 average
No. of survived explants						
2	Kh	3.00^{c}	2.00^{d}	3.00^{c}	3.00^{c}	2.75
	Ru	3.00^{c}	4.00^{b}	5.00^{a}	5.00^{a}	4.25
	Sh	4.00^{b}	5.00^{a}	2.00^{d}	2.00^{d}	3.25
4	Kh	3.00^{b}	3.00^{b}	2.00^{c}	3.00^{b}	2.75
	Ru	3.00^{b}	3.00^{b}	4.00^{a}	4.00^{a}	3.50
	Sh	3.00^{b}	4.00^{a}	4.00^{a}	4.00^{a}	3.75
6	Kh	3.00^{d}	4.00^{c}	4.00^{c}	5.00^{b}	4.00
	Ru	5.00^{b}	5.00^{b}	6.00^{a}	5.00^{b}	5.25
	Sh	6.00^{a}	6.00^{a}	4.00^{c}	4.00^{c}	5.00
8	Kh	3.00^{d}	2.00^{e}	4.00^{c}	5.00^{b}	3.50
	Ru	5.00^{b}	5.00^{b}	6.00^{a}	5.00^{b}	5.25
	Sh	6.00^{a}	6.00^{a}	4.00^{c}	4.00^{c}	5.00
CV average		3.92	4.08	4.00	4.08	
Browning initiation						
2	Kh	1.00^{a}	1.00^{a}	1.00^{a}	1.00^{a}	1.00
	Ru	1.00^{a}	1.00^{a}	1.00^{a}	1.00^{a}	1.00
	Sh	1.00^{a}	1.00^{a}	1.00^{a}	1.00^{a}	1.00
4	Kh	2.00^{c}	2.00^{c}	2.00^{c}	2.00^{c}	2.00
	Ru	3.00^{b}	2.00^{c}	3.00^{b}	3.00^{b}	2.75
	Sh	4.00^{a}	3.00^{b}	3.00^{b}	3.00^{b}	3.25
6	Kh	2.00^{b}	3.00^{a}	1.00^{c}	2.00^{b}	2.00
	Ru	3.00^{a}	2.00^{b}	3.00^{a}	3.00^{a}	2.75
	Sh	3.00^{a}	3.00^{a}	3.00^{a}	3.00^{a}	3.00
8	Kh	3.00^{a}	3.00^{a}	1.00^{c}	2.00^{b}	2.25
	Ru	3.00^{a}	2.00^{b}	3.00^{a}	3.00^{a}	2.75
	Sh	3.00^{a}	3.00^{a}	3.00^{a}	3.00^{a}	3.00
CV average		2.42	2.17	2.08	2.25	
Callus formation						
2	Kh	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00
	Ru	2.00^{a}	1.00^{b}	1.00^{b}	1.00^{b}	1.25
	Sh	2.00^{a}	2.00^{a}	1.00^{b}	1.00^{b}	1.50
4	Kh	2.00^{b}	2.00^{b}	1.00^{c}	1.00^{c}	1.50
	Ru	2.00^{b}	1.00^{c}	1.00^{c}	1.00^{c}	1.25
	Sh	1.00^{c}	3.00^{a}	1.00^{c}	1.00^{c}	1.50
6	Kh	2.00^{b}	2.00^{b}	1.00^{c}	1.00^{c}	1.50
	Ru	2.00^{b}	1.00^{c}	1.00^{c}	1.00^{c}	1.25
	Sh	1.00^{c}	4.00^{a}	1.00^{c}	1.00^{c}	1.75
8	Kh	1.00^{e}	1.00^{e}	3.00^{c}	3.00^{c}	2.00
	Ru	5.00^{a}	2.00^{d}	3.00^{c}	3.00^{c}	3.25
	Sh	2.00^{d}	4.00^{b}	2.00^{d}	2.00^{d}	2.50
CV average		1.92	2.00	1.42	1.42	

*p-values ≥0.05 are significant

the availability of different nutrient media for tissue culture technique, MS medium is generally the best for micropropagation and active constituents formation as well as B5 medium (Gamborg *et al*., 1968) as it increases the ratio of acidic compounds formation by 15% than for traditionally cultured plant in the field (Zenk *et al*., 1977) in a period of time extremely lower than the naturally grown plants. This is the reason that most pharmaceutical companies found a tissue culture unit for cultivating the plants that contain those important compounds which save more efforts and time as well as hard currency required for importing those important compounds in addition to providing those important compound all around the year (Al-Turki *et al*., 2010; Alturki *et al*., 2013). Many investigators obviously showed the important role of MS in explant growth and division for callus initiation, in embryogenesis induction and buds formation stages followed by SH medium (Schenk and Hildebrandt, 1972) to a lower extent than B5 medium and at last the ER medium (Eriksson, 1965). Also, Mohamed (1996) observed that the diversity of nitrogen sources in the medium greatly helps in callus formation. While, Bekheet and Saker (1998) declared that high concentrations of auxins are required for the initiation of callus formation from the explants that is consistent with the findings of Letouze *et al*. (2000).

In general, the nutrient medium contains nitrogen at a concentration of 25-60 μM. Since plant cells consume nitrogen in the form of either nitrates or ammonium and the previous studies proved that the addition of both nitrates and ammonium together in the nutrient medium increases the growth rate of the cultured tissues. It was also found that the optimum concentration of nitrates in

the medium ranges from 25-40 μM. It has been observed that usually date plants prefer nitrogen in ammonium form rather than the nitritic form. While, the ammonium ranges from 2-20 μM in easy absorption form in the nutrient medium than nitrates (Al-Marri, 1995). Therefore, the ionic balance between NH_4^+ and NO_3^- in the nutrient medium is an important factor affecting the success of any *in vitro* or *in vivo* propagation of any plant. As for as the physiological role of nitrogen is concerned, it is involved in amino acids structure that forms protein, a basic structure of cells. It is also involved in vitamins, nucleic acids, enzymes and hormones structure that help in completing the photosynthesis process in addition to its important role in the formation of chlorophyll, alkaloids, phenols and many others important secondary compounds and antioxidants (Asemota *et al*., 2007; Nadeem *et al*., 2011; Alturki *et al*., 2013).

Nitrogen is considered as one of the most important essential inorganic nutrients needed by the plant for its growth and development due to its role in completing the metabolic processes affecting the formation of secondary products and antioxidants either directly or indirectly as proved by the previous studies. Similarly, a recent study concluded that the rate of formation of antioxidants differ from plant to plant, cultivar to cultivar, according to the age of tree, its growth season, plant physiology and the type of explants used (Shehata *et al*., 2014).

Normally, ammonium nitrate (NH_4NO_3) and potassium nitrate (KNO_3) are added to the nutrient medium as an important source of inorganic nitrogen for tissue culture (Shehata *et al*., 2014). Besides, some researchers preferred using organic instead of inorganic nitrogen in the form of asparagine or glutamine (Raghavan *et al*., 1976; Mok *et al*., 1978; Abo-El-Nil, 1986). The previous studies reported that nitrogen positively affect some metabolic processes in plants that increase growth. It also activates the enzymes responsible for chemical reactions as catalysts required for active constituents and secondary products formation during cell division like RNA polymerase enzyme (Morre and Cherry, 1977; Murray and Key, 1978).

With respect to potassium, it is considered as one of the most important mobile monocations in supporting and transporting cells of plant like xylem and phloem in plant root and stem present in high concentrations upto 200 μM. It is also essential for completing the enzymatic processes of the plant. It is generally added to the nutrient medium in the form of KNO_3 or KH_2PO_4 with a concentration of 1-3 μM and rarely added in the form of KCl (Al-Marri, 1995). Potassium (K^+) like calcium (Ca^{+2}), sodium (Na^+) and phosphorus (P^-) is one of the most important ion for growth and development of plant in general and cells and tissue in particular (Al-Whaibi and Basala, 1986; Al-Whaibi, 1997). In other studies (Al-Whaibi, 1990, 2009) mentioned that metabolic processes differ from cultivar to cultivar, tree to tree, also according to the surface area of the leaf and its age, oxidation and reduction rates and subsequently the rates of formation of phenolic compounds differ according to the cultivar of date palm.

The study results showed the importance of both using nitrogen with its optimum concentration (full KNO_3) in addition to keeping balance between auxins and cytokinins for callus formation and characterization of the cultivars under study. As both the nitrogen sources (represented in ammonium nitrate) and the growth regulators play a catalytic 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. These results agree with those of Tisserat (1979) and Mohamed *et al*. (2001) who stated that the type and concentration of auxin play an important role in callus formation. Similar results were reported by many researchers who found that different cultivars affect the callus initiation, browning occurrence, phenolic compounds and antioxidants (Bhargava *et al*., 2003; Eshraghi *et al*., 2005).

For a long time, it was thought that one of the major disadvantages of micropropagation of any plant *in vitro* is the formation of phenolic compounds by the explants that result in turning the whole colour of the explants tissue into brown then black eventually leading to its death and is called as the browning phenomenon after detecting the important role of phenolic compounds in the formation of antioxidants. Previously, more attention was paid to date palm fruit as an important source of essential antioxidants for human health (Drira and Al-Sha'ay, 1993). Browning phenomenon is usually attributed to the formation of phenolic compounds which turns to highly toxic quinones in presence of oxidation enzymes like poly phenol oxidase and peroxidase. These enzymes are characterized by their copper content which leads to browning of the explant. Generally, the colour gradually changes from yellow to dark brown then black after the formation of phenolic compounds. Similar results were reported by many researchers who found that disvitality and mortality of the plant is mainly due to the interaction between phenols and proteins which leads to disactivation of many important enzymes for the plant (Zaid, 1984, 1989a; Alturki *et al*., 2013; Shehata *et al*., 2014).

Table 3: Effect of the potassium nitrate (KNO_3) strength on total phenolic content and antioxidant production

Cultivars date palm and strength KNO_3	Total phenolic (mg g^{-1}) as express a Gallic acid	Antioxidant activity by ABTS	
		Inhibition (%)	UM Trolox
Khalas			
Zero	1.156±0.003^{d}	47.133±0.214^{d}	436.892±3.948^{d}
Half	0.531±0.003^{j}	21.373±0.221^{j}	157.566±1.987^{j}
Full	0.998±0.020^{e}	42.853±0.090^{e}	391.654±1.458^{e}
Double	1.192±0.004^{c}	48.269±0.016^{c}	449.764±1.510^{c}
Ruziz			
Zero	0.638±0.004^{h}	20.316±0.718^{k}	137.945±3.056^{k}
Half	0.557±0.006^{i}	23.332±0.020^{i}	177.784±0.375^{i}
Full	0.653±0.002^{h}	28.945±0.197^{h}	233.401±3.001^{h}
Double	0.522±0.03^{j}	21.617±0.01^{j}	159.119±1.059^{j}
Shishi			
Zero	1.647±0.005^{b}	66.821±0.397^{b}	651.579±2.497^{b}
Half	2.053±0.010^{a}	80.694±0.439^{a}	801.575±2.391^{a}
Full	0.955±0.010^{f}	39.324±0.287^{f}	359.867±5.173^{f}
Double	0.819±0.004^{g}	33.426±0.03^{g}	287.733±3.448^{g}
LSD at 0.05	1.754	1.754	0.404

It has been proven that some minerals and inorganic compounds added to the nutrient medium may cause some problems that greatly enhances browning emergence while there are other minerals and compounds that can greatly limit or prevent this phenomenon. For example, the high concentration of NH_4^+ in the nutrient medium increases medium acidity and decreases potassium absorption thus increasing the formation of phenolic compounds which greatly lower the formation of embryonic callus in date palm resulting in browning of the explants. When the concentration of $KH_2PO_4.2H_2O$ exceeds 200 mg L^{-1}, browning phenomenon occurs that is why, the balance between NH_4^+ and NO_3^- should always be maintained as well as between NO_3^- and K^+ in the nutrient medium to overcome this phenomenon (Al-Marri, 1995). There are other factors that highly support browning occurrence as stated by Zaid (1984). These are high light intensity, high temperature, excessive use of sterilizers and plant hormones specially auxins due to explant´s injuries and because of some factors related to the explant itself such as age of the mother plant and the time at which the explants are taken (Ibrahim *et al*., 1999).

Some previous studies indicated that phenolic compounds play a growth inhibitory role while acting as anti-auxins and at the same time protect the natural auxins from deterioration i.e., the co-auxin and this contradictory effect explains the conflicting physiological activity on the explant. The studies also monitored the role of some active phloroglucinol on explants rooting *in vitro* as in the case of apple (Jones *et al*., 1979; James *et al*., 1981; Rao and Ravishankar, 2002).

Effect of the potassium nitrate (KNO_3) concentration on total phenolic contents and antioxidants production: Data in Table 3 show the combined effect of potassium nitrate concentration (KNO_3) and the three date palm cultivars. The highest mean phenolic contents (expressed as gallic acid contents in regenerated callus tissues) were 1.1923±0.004 mg g^{-1} in Khalas cv. In extracts cultured in double MS and 0.6538±0.002 and 2.0531±0.01 mg g^{-1} in Ruiziz with full concentration and in Shishi with half concentration of potassium nitrate (KNO_3).

The ABTS inhibitory activity of adventitious callus tissues decreased by using half concentration of potassium nitrate with Khalas cv. It was 20.316 when using zero concentration with Ruiziz cv. On the other hand, double concentration gave 0.819 with Shishi cv. The study results agree with other researchers who reported a high positive correlation between free radial scavenging activity and the total concentration of phenolic compounds in plant (Zheng and Wang, 2001; Wangensteen *et al*., 2004; Tawaha *et al*., 2007; Alturki *et al*., 2013; Shehata *et al*., 2014). Also, a positive correlation was observed between the antioxidant activity and the total phenolic content for methanolic extracts of 51 Jordanian plant species including *P. dactylifera* explants. A relatively similar graduation was previously observed for other explants (Lee *et al*., 2011; Sae-Lee *et al*., 2011). Nitrogen sources are important for secondary product synthesis of compounds such as alkaloids (Zhong, 2001), anthocyanin's and shikonin from cell suspension cultures (Kim and Chang, 1990). Furthermore, the half concentration of KNO_3 produced the maximum value of phenolic contents thus causing the highest browning of callus tissues for all the cultivars. 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 formation of embryonic callus (Zaid and Arias-Jimenez, 1999;

Al-Ibresam and Al-Meer, 2008). This is further supported by the positive result obtained by Sharma *et al*. (1984) who reported limiting browning phenomenon by increasing the $KH_2PO_4.2H_2O$ concentration. Also, it is important to mention that high concentration of plant hormones like auxins and BAP greatly increase the production of phenolic compounds (Zaid, 1989b).

CONCLUSION

This study on cultures of cell suspensions of date palm (*Phoenix dactylifera* L.) showed that secondary metabolites accumulation was influenced by potassium nitrate. The highest accumulation of phenolic contents and antioxidants activity were found in the medium with half concentration of KNO_3. Consequently, the phytochemical quality of regenerated explants can be increased as a result of their higher content in phenolic compounds with antioxidant activity.

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