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BOTANICAL STUDIES ON THE MICROPROPAGATION OF DATE PALM (*Phoenix dactylifera,* L.) USING TISSUE CULTURE TECHINIQUE:

I- EFFECTS OF ANTIOXIDANT; AOS ON BROWNING CONTROL AND RELATED COMPOUNDS.

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ABSTRACT

Antioxidants; ascorbic and /or citric acids in Bartamoda and sakkoty cv(s) increased F.Wt of the initial explant, its growth value and total phenols whereas decreased browning degree of the tissues and the adjacent medium as well as the concentrations of H_2O_2 and proline. AOs increased the concentrations of AsA and GSH in the developed explant of both date palm (*Phoenix dactylifera L*,) cv(s). AsA showed an additive effects to that of citric on increasing non-enzymatic and enzymatic antioxidants concentrations in both cv(s) .

Bartamuda cv showed, in general, higher F.Wt as well as concentrations of total phenols, H_2O_2 and proline , than Sakkoty cv. However,concentrations of AsA and GSH as well as CAT, GPOD and SOD in Baramuda cv was found to be less than that of Sakkoty cv over all treatments. This indicated that Bartamuda cv has high ability to used AsA and GSH specially the latter, in the synthesis of thio containing molecules, particularly salfhydryl-containin amino acids.

Keywords: Phoenix dactylifera – Proline - Reduced glutathione - Guaiacol peroidase -Superoxide dismutase

INTRODUCTION

Date palm (*Phoenix dactylifera,* L ; Palmaceae, Arecaceae) micropropagation has been initiated to produce plantlets from economically important cultivars such as Bartamuda , Sakkoty and others. It has been considerable interest in developing tissue culture micropropagation techniques to obtain large number of plantlets and reduce the dependence on offshoots propagation. Tisserat (1979 a and b) found that, date palm tissues are especially susceptible to lethal browning substances discharged from the explants which withered and died (Khalil *et al.,* 1983). The cultured explants on nutrients medium, release toxic substances such as phenolic compounds which induce rowning and inhibit tissues growth. Thereafter, the explants deteriorate and go to die. due to a conjugation between phenols and the protein. Subsequently, the enzymes loss their activity (Nash and Davies, 1972). They added that, the increase in phenolic compound production has been associated with a decrease in growth and a decline in protein synthesis. Phenolic compounds oxidized and become quinones (toxic compounds) by the enzymes polyphenol oxidase and peroxidase (Hu and Wang, 1983). These quinones make browning of the tissues, begin from yellow to dark brown to black colour and lead it to die.

The increase in the production of phenolic compounds has been associated with high levels of exogenous sugars (Rhodes and Wooltoron 1978). Hegazy (2003) found a highly significant interaction between the concentration of auxin and sucrose as well as phenolic compounds in the media. Grambow and Langenbeck-Schwich (1983) reported that, the substitution pattern of phenols (auxin protectors) affects the rate of IAA degradation. Some substituted monophenolic increased its rate whereas others depressed it.

To minimize browning ,Forrest (1969) indicated that, light stimulated flavonoid synthesis in tea (*Camellia sinensis,* H.). and reduced the production of phenols and browning as well as acid culture establishment. Similar results were reported by El-Shafey *et al* (1999) on date palm Siwi cv. Tisserat (1984 a&b) investigated the anti-browning effect of a number of substances such as ascorbic acid, dihydroxynaphthalene, dimethyl sulfoxide and PVP but he found that, all the previous compounds were not effective. Zaid (1986) reported that, the browning of tissues may be prevented by presoaking explants in antioxidant solution of 150 mg/l citric and 100 mg/l ascorbic acid, as well as by using small explants and subculturing on fresh medium for short period of incubation. Pierik (1989) reported that, the most important aspects of A. C was adsorption of toxic brown/black pigments (phenol-like compounds, and melanin) and other unknown colorless toxic compounds and this in turn, might promote somatic embryogenesis. Similarly Ibrahim and Hegazy (2001 a&b) mentioned that, addition of A. C. to date palm culture medium at the level of 1.5 g/l was superior for minimizing browning.

Some researchers who works on date palm tissue culture, embloyed some nutrients, amino acids and PVP to decrease browning. Apavatjrut and Black (1977) and Hegazy (2003) suggested that, browning could be eliminated by employing a nutritionally balanced medium. Lee and Kirby (1986) reported that, when *Pseudotsuge menziesii* grown on glutamine at concentrations 10, 30 and 50 mM, cell cultures grew rapidly with a shortened log phase. Bekkaoui *et al.* (1987) found that, the addition of 5mM glutamine to the cell suspension culture medium of *Picea glauce* were necessary for callus formation. Pierik (1987 and 1989) suggested that, the addition of three amino acids (glutamine, argeninine and asparagines) may minimize browning. Similarly, Suprasanna *et al.* (1994) found that, L - proline and L-glutamine increased the frequency of embryogenic callus production and ecreased browning. Prathanturarug *et al* (1996) studied the effects of antioxidants, absorbents, light and dark conditions on browning phenomenon and found that, PVP-10 at 1 g/l reduced browning of *Nees* explants. They added that, citric acid at 50 mg/l increased shoot elongation and showed an antibrowning properties. Similarly, activated charcoal decreased browning but caused some hyperhydricity symptoms. Incubation under low light intensity at 600 Lux gave the best antibrowning effect. However they repoted that, pretreatment with antibrowning before incubation has no effect on reducing browning.

The present investigation aimed to study the effects of antioxidant; AOs on browning control and related compounds

MATERIALS AND METHODS

The present investigation was carried out at the plant tissue culture Dept .,Genetic of Engineering and Biotechnology Research Institute(G E B R I) EL-Sadat city ,Menofia Unvi. Egypt and the Laboratories of Agric.Bot.Dept., Faculty of Agric. Mansoura Univ. Egypt, during the period of 2003 – 2007.

Femal date palm (*phoenix dactylifera,* L),Bartamuda and Sakkoty cv(s), which commonly known as an important dried cv(s) grown at Aswan governorate ,Egypt were used to exploring the alleviation effects of antioxidant on controlling browning, as well as related compounds .

Explants from each cv(s) were prepared as previously maintained (El-Hosiney Hanan, 2008) and cultured in MS basal nutrient media (Table 1) with or without ascorbic acid (75 ml/l), citric acid (75 ml/l) and ascorbic + citric.(75+75 mg/l). Gelrite as a gelling agent at 1.5 g/l was used. The pH was adjusted at 5.7-5.8 prior to the addition of gelrite.

Each treatment was replicated 6 times (6 culturing jars; 250 ml.). The nutrient media for each treatment was distributed into culture jars, each one contained 35 ml of the specific prepeared medium.

The culture jars were immediately capped with polypropulin closure and autoclaved at 121^{o}C, 15/1bs/inch^{2} for 20 minutes.

Sterilized shoot tip explants were cultured on the specific medium and incubated at 25-27^{o}C for 16/18 hrs day/night condition using white fluorescent tubes giving intensity of about 1500 Lux. The incubation was took place for 6 months with 4-sub-culturing, 6 weeks intervals. At each sub-culturing date, all survived plants were transferred and recultured on the same fresh specific media and the number as well as percentages of shoot tip explants which produced axillary buds were recorded.

To prepare stock solution F: dissolve each constituents in 200 ml distilled water; heat Na_2EDTA solution; with continuous stirring add Fe $SO_4 7H_2O$ solution when cool dilute to 1000 ml with distilled water

Myo-Inosito; 1 g/l, Biotin; 0.2 mg/l, Asparagen; 125 mg/l, Glutamine; 200 mg/l and Adenine Sulfate ;20 mg/l were added respectively to the MS medium as suggested by the authoress (El-Hosieny, Hanan, 2002).

At the end of incubation period, 6 months, the following data were recorded : - Browning (Pottino 1981) and Fresh weight; F.Wt of the developed exlpant, Concentrations of H_2O_2 (Velikova *et al*, 2000)., Ascorbic acid (Cakmak and Marschner 1992) and Glutathione (GSH) (Abd El-Salam, Heba 2006) were determined. The activity of the antioxidant enzymes: catalase; CAT (Velikova *et al*, 2000) , guaiacol peroxidase; GPOD (Urbanek et al, 1991) and, superoxive To prepare stock solution F: dissolve each constituents in 200 ml distilled water; heat Na_2EDTA solution; with continuous stirring add Fe $SO_4 7H_2O$ solution when cool dilute to 1000 ml with distilled water

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dismutase ;SOD (Van Rossun *et al* 1997) as well as concentrations of proline: (Bates *et al*, 1973),and total soluble phenols (Swain and hills , 1959 and Danial and George, 1972) were also determined.

All data were subjected to statistical analysis according to(Gomez and Gomez 1984).

Table 1 :Composition of basal nutrient medium of Murashige and Skoog; MS (1962).

Stock solution S.S.	Constituents	g/liter S.S.	mg/liter medium	To make up liter of MS medium ml
A	NH_4NO_3	82.5	1650	20
B	KNO_3	95.0	1900	20
C	H_3BO_3	1.24	6.2	5
	KH_2PO_4	0.05	170	
	KI	34.0	0.83	
	$Na_2MoO_42H_2O$	0.005	0.25	
	$CoCl_22H_2O$	0.166	0.025	
D	$CaCl_22H_2O$	88.00	440	5
E	$MgSO_47H_2O$	74.00	370	5
	$MnSO_44H_2O$	1.72	22.3	
	$ZnSO_47H_2O$	4.46	8.6	
	$CuSO_45H_2O$	0.005	0.025	
F	Na_2EDTA	7.45	37.25	5
	$FeSO_47H_2O$	5.57	27.85	
G	Thiamine HCl	0.2		5
	Nicotinic acid	0.1		
	Pyridoxine HCl	0.1		
	Glycin	0.4		

Stock solution F: is made differentially from the others.

RESULTS AND DISCUSSION

Data in Table 2 show that F. Wt of the initial explant, growth value (data not presented) and total phenols were increased whereas browning degree of the tissues and the adjacent medium as well as the concentrations of H_2O_2 and proline were decreased due to AOs application. The decrease was more pronounced in response to ascorbic + citric acids treatment. in both genotype of date palm. The presence of the two antioxidants recorded additive effects in this respect in both genotypes. There is no significant difference between the two AOs used in both cv(s). Browning may be due to the oxidation of polyphenols and the formation of quinones which are highly reactive and toxic to date palm tissues. El-Meskaoui and Tremblay (2001) suggested that ethylene could cause tissue browning, it activate the synthesis of oxidative enzymes or inhibit the synthesis of protective enzymes. In addition, `date in the present investigation

show that over all treatments, Bartamuda cv showed, in general,high F.Wt as well as concentrations of total phenols H_2O_2 and proline values compared with Sakkoty cv. The lowest F.Wt values was noticed in MS

treatment with Sakkoty cv compared with the corresponding decrease noticed in Bartamuda cv. In addition, the highest reduction in the concentrations values of H_2O_2 and proline were recorded in the presnce of ascorbic and citric acids together. It seems also that, ascorbic acid was more effective in this respect.

Table 2:Effects of MS media with or without antioxidants; AOs on fresh weigh; F.Wt (g/explant) of the explant at the end of callus stages (24 weeks), Browning degree, enzymatic and non enzymatic antioxidants as well as certain other browning- related metabolites.

Gen-otype cv(s)	Treatments mgl^{-1}	F.Wt g/jar	Browning degree	TSP	H_2O_2	Proline	SOD	AsA	GSH	CAT	GPOD
Barta muda	MS Control	5.2	4	0.8	1.07	1.59	150	250	50.3	70	8
	MS+Ascorbic	6.0	3	1.1	0.61	0.15	106	317	74.9	99	10
	MS+Citric	6.2	3	1.1	0.60	0.75	110	333	68.0	92	9
	MS+AsA+Cit	7.8	2	1.8	0.56	0.60	206	360	86.5	110	13
	Mean	6.3	3	1.2	0.71	0.77	143	315	63.18	9275	10
Sakko ty	MS control	5.08	4	0.6	1.03	0.78	250	300	66.6	107	13
	MS+Ascorbic	5.40	3	0.8	0.60	0.44	256	330	80.3	110	13
	MS+citric	5.44	3	0.9	0.57	0.44	264	346	86.8	120	14
	MS+ASA+Cit	6.14	2	1.1	0.50	0.38	270	383	103.9	137	15
	Mean	5.52	3	3.1	0.68	0.51	260	339.75	84.4	118.5	13.75
Mean	MS Control	5.14	4	0.7	1.05	1.19	200	275	58.45	88.5	10.5
	MS+Ascorbic	5.7	3	0.95	0.61	0.30	181	323.5	77.6	104.5	11.5
	MS+Citric	5.82	3	1.00	0.59	0.60	178	339.5	77.4	106.0	11.5
	MS+AsA+Cit	6.97	2	1.45	0.53	0.49	238	371.5	95.2	123.5	14.0
N-LSD at 5% for:											
Genotype; A		**0.07**		**0.02**	**0.03**	**0.01**	**2.0**	**4.2**	**3.5**	**1.2**	**0.07**
Treatments; B		**1.08**		**0.02**	**0.04**	**0.01**	**2.6**	**4.5**	**3.7**	**1.3**	**0.09**
Genotype x treatments (AxB)		**1.07**		**0.03**	**0.06**	**0.02**	**3.1**	**6.1**	**4.1**	**1.6**	**1.10**

F.Wt = Fresh weight (g / explant) **Browning degree according to Pottino (1981)**
TSP = Total soluble phenols (mg/g F.Wt) **H_2O_2 = Hydrogen peroxide (µM g^{-1} F.Wt)**
Proline (mg g^{-1} F.Wt) **SOD = Superoxide dismutase (µg g^{-1} F.Wt)**
AsA = Ascorbic acid (µg g^{-1} F.Wt) **Cit = Citric acid**
GSH = Reduced glutathione (µg g^{-1} F.Wt) **CAT = Catalase (µg g^{-1} F.Wt)**
GPOD= Guaiacol peroidase (µg g^{-1} F.Wt)

The increasing effects of AOs on F.Wt may be due to their antioxidative effects on increasing mitotic activity (Jiang *et al*, 2001, and Munne-Bosch, 2005). A specific roles of the AOs used were previously reported by Abdel-Salam, Heba (2006) who reported that AOs many contribute to their alleviation effect and the role of reduced glutathione. He added that, the alleviation effect of AsA may be due to its enhancing effect on cell division and synthesis of hydroxy-proline-rich protein. Wingate *et al*, (1988) found that AOs regulated the gene expression and being the precursor of phytochelatins

The increasing effects of AOs on total phenols may be due to their effects on reduction the phenollic compounds oxidation (Murashige, 1974). Ichihashi and Kako(1977) found that AOs did not prevent phenols oxidaton

but prevent quinones polymerization and reduced the probability to react wih protein. The reduction effects of AOs on brwning as well as the negative relationships between browning degree and total soluble phenols were previously mentioned (Hegazy, 2003)

It was found that phenolic compounds was essential for multiplication and rooting in micropropagation of many plant species (Bekheet *et al,*2008). Al-Khateeb (2008) reported that browning may be due to the oxidation of the various wounding exudates polyphenols compounds to form quinones, highly reactive compounds which polymerize rapidly with protein by covalent bonds which are toxic for date palm tissue growth and development. In intact tissue, phenolic compounds were apparently situated in separate pools or compartments within the cells. These pools are integrated with wounding and oxidized. The inhibitory action of phenols may results from its oxidation to quinones by poly phenols oxidase and peroxidase and subsequent binding with protein, such process may lead to the loss of various enzyme activities (Hu and Wang, 1983). Hegazy (2003) reported that, phenolic exudate released compounds were decreased from the explants after 24 h in the media containing high level of ascorbate and citrate.

Increasing H_2O_2 as well as other reductive oxygen species; AOs leading to oxidative stress is a principal component of their damaging effect on plant tissues (Schutzendubel and Polle, 2002).

The protective role of AOs against H_2O_2 accumulation was previously reported (Cheng, 2003; Guo *et al*, 2005 and Munne-Bosch, 2005) . According to Abd El-Slam, Heba 2006), AsA reduced glutathione; GSH and ∝-tocopherol ; T have each been shown to act as antioxidants in the detoxification of reactive oxygen species; ROS in aerobic cells. They have central and interrelated roles acting both chemically and as substrates in enzyme-catalyzed detoxification reactions. Ascorbic acid; AsA is an important compound of the plant antioxidant defence system and serves as a reductant for the peroxidative removal of H_2O_2 **.** Reduced glutathione; GSH directly reduces most of reactive oxygen species; ROS and maintain the accorbate pool in plant cell. Munne-Bosch, (2005) added that under stress conditions, -tocopherol; T prevents the propagation of lipid peroxidation by scavenging reactive oxygen species; ROS .

The reduction effects of AOs on proline concentration may be due to their stress alleviation effects (Abd El-Slam Heba, 2006) in addition to their enhancing effects on cell division (Sanchez-Fernandez, *et al*, 1997). These effects leed to an acceleration of proline consumption in the synthesis of hydro-xyproline-rich proteins which are necessary for progression through the cell cycle (Arrigoni *et al* ,1992).

Data tablueted in the same table show also that AOs increased the concentrations of AsA and GSH in the developed explant of both date palm (*phoenix dactylifera L*,) cv(s). The enhancing effects of ascorbic acid treatment was less than those of citric acid treatment on AsA and GSH concentrations. AsA showed an additive effects to that of citric on increasing non-enzymatic antioxidants concentrations in both cv(s) ; since treatment MS+ cltric showed highest values in this respect.

Antioxidants (AsA and/or citric,) increased the activity of the antioxidant enzymes in the developed explants in both genotypes date palm. Ascorbic + Citric acids treatment led to the highest induction in the activity of all enzymes estimated.

The interaction effects between the genotype and the AOs treatments on antioxidants enzymes activaties were significant. These results indicated that AOs induced oxidative stress. Smeets *et al*, (2005) reported that, plant cells respond to elevated levels of oxidative stress by activating their antioxidative defence system and the first group of enzymes involved in this defence are the ROS- quenching enzymes such as, CAT, PODS and SOD. Cheng (2003) recorded that, CAT, PODS and SOD are important enzymes for plant adaptation to environmental stresses as the harmonious interaction of the three enzymes make the balance between ROS production and elimination, thus keeping the level of ROS in plant tissues low, to prevent the injury of cells. In peroxisomes, H_2O_2 can be destroyed by CAT. CAT produces molecular oxygen and water from two molecules of H_2O_2. Since these two molecules must impinge simultaneously at the active site, CAT has a very high maximum velocity. PODS is an important role in scavenging H_2O_2 and organic peroxidase (Smeets, *et al,* 2005). Moreover, Abd El-Salam, Heba, (2006) reported that SOD causes the catalytic dismutation of potentially toxic superoxide anion radical (O^{2-}) to H_2O_2 whereas, CAT decomposes H_2O_2 to water and oxygen molecule, both enzymes provide an efficient mechanism for the removal of free radicals from the cells .

It has been concluded that, controlling browning phenomenon is a general goal by tissue culture workers. Inducing antioxidative enzyme activities is important to overcome oxidative stress due to the imposition of environmental stress (Foyer *et al*, 1994). This induction may be due to an enhanced gen (s) activity, since overexpression of genes encoding these enzymes in several transgenic plant species confers protection aganest free radical-induced oxidative stress (Abd El-Salam, Heba, 2006).

Data also show that, the values of ASA and GSH concentrations as well as CAT, GPOD and SOD in Baramuda cv was found to be less than that of Sakkoty cv over all treatments. This indicated that Bartamuda cv has high ability to used AsA and GSH specially the latter, in the synthesis of thio containing molecules, particularly salfhydryl-containin amino acids; A A GSH and PG. Cysteine is required for GSH synthesis which, in turn, is required in PCs synthesis . PCs are able to create complex compounds with toxic ions (Piechalka *et al*, 2002) transported into the vacuole by the ABC transporter which is localized in the tonoplast (Oritz *et al*, 1995) thus separating them from cell metabolism.

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دراسات نباتية على الاكثار الدقيق لنخيل البلح باستخدام تقنية زراعة الانسجة:
١- تـاثيرات مضـادات الاكسـدة علـى الـتحكم فـى ظـاهرة التلـون البنـى والمركبـات المرتبطة ذات العلاقة.

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أدى استخدام أى من حمض الستريك، أو حمض الأسكوربيك، أو كلاهما معا، الى تقليل درجة التلون البنى للمنفصلات النباتية المستخدمة لكلا صنفى نخيل البلح وبيئتها المحيطة.و كان ذلك مصحوبا بخفض تركيز فوق أكسيد الهيدروجين والبرولين، مع زيادة الوزن الغض للمنفصلات النباتية، ودليل النمولها، وتركيز الفينولات الكلية . ولقد تفوق صنف البرتمودا عن السكوتى فى قيم الوزن الغض، وتركيز الفينولات، وفوق أكسيد الهيدروجين، والبرولين .كما زادت تركيزات مضادات الأكسدة الغير إنزيمية والإنزيمية و حمض الاسكوربيك ،و الجلوتاثيون فى المنفصلات النباتية لكلا الصنفين، كنتيجة مباشرة، لإضافة حمض الستريك أو الأسكوربيك الى البيئة المستخدمة، وكان لإضافتهما معا أثر إضافى فى هذا الشأن.

و لقد تميز صنف السكوتى عن البرتمودا بإرتفاع قيم تركيزات حمض الاسكوربيك، و الستريك ، الجلوتاثيون ، و نشاط انزيمات الكتاليز، والبيروكسيديز تحت تأثير نفس المعاملات، مما يشير الى قدرة الصنف على إستخدام الاسكوربيك ،و الجلوتاثيون – وخاصة- الأخير فى تخليق الجزيئات المحتوية على مجموعات كبريتيةن وخاصة الأحماض الأمينية منها .