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Growth, lipid peroxidation, organic solutes, and anti-oxidative enzyme content in drought-stressed date palm embryogenic callus suspension induced by polyethylene glycol

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Abstract:	This study aimed to evaluate the effects of polyethylene glycol (PEG) on growth synchronization of embryo development in date palm cell suspension and the effect on lipid peroxidation, organic solute content and antioxidant enzymes activities. Callus maintained on MS basal media was transferred to regeneration liquid media supplemented with increasing levels (0-20%) of polyethylene glycol 6000 (PEG) to induce osmotic stress. Maximum values of the degree of embryogenic callus formation and its fresh weight as well as the percentages of normal embryos callus shapes were increased whereas abnormal embryogenic callus was decreased with an increase in the level of PEG up to 10% and thereafter decreased. Organic solutes estimated represented by total soluble protein (TSP), proline, glycine betaine (GB), total soluble phenol (TSPh), total sugars (TS) and total soluble organic acids (TOA) were increased whereas superoxide dismutase (SOD) activity was decreased due to PEG supplementation up to 15% and thereafter decreased. Raising the PEG level increased malondialdehyde (MDA) concentration and SOD activity up to 10% PEG and thereafter decreased. However glutathione reductase (GR) and catalase (CAT) activities were decreased in general at the highest levels of PEG. Proliferation of somatic embryos was influenced by their developmental shapes. The proportion of normal embryo developmental shapes were about 50% compared with 20% abnormal shapes at optimum levels of PEG. Cultivar (cv.) Samani accumulated more organic solutes compared cv. Sewi under normal and stress inducing media. In contrast, lipid peroxidation, GR, SOD and CAT activities were significantly higher in cv. Sewi than in cv. Samani.

	These results indicate that the cv. Samani has the ability to tolerate a higher level of drought stress compared to cv. Sewi due to the osmotic re-balancing within its tissues.
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Running Title: PEG induced antioxidant changes in date palm embryogenic callus

Contributions of the Authors:

M N H – jointly developed the proposal for the research, designed and prepared the methodology and wrote the results discussion
ARHEH - jointly developed the proposal for the research, designed and prepared the methodology, collected data and carried out the statistics
NMES - jointly developed the proposal for the research, undertook chemical analysis, collected data and carried out statistics and wrote the bulk of the manuscript
MPF – responsible for oversight of research and for manuscript editing

ABSTRACT

This study aimed to evaluate the effects of polyethylene glycol (PEG) on growth synchronization of embryo development in date palm cell suspension and the effect on lipid peroxidation, organic solute content and antioxidant enzymes activities. Callus maintained on MS basal media was transferred to regeneration liquid media supplemented with increasing levels (0-20%) of polyethylene glycol 6000 (PEG) to induce osmotic stress. Maximum values of the degree of embryogenic callus formation and its fresh weight as well as the percentages of normal embryos callus shapes were increased whereas abnormal embryogenic callus was decreased with an increase in the level of PEG up to 10% and thereafter decreased. Organic solutes estimated represented by total soluble protein (TSP), proline, glycine betaine (GB), total soluble phenol (TSPh), total sugars (TS) and total soluble organic acids (TOA) were increased whereas superoxide dismutase (SOD) activity was decreased due to PEG supplementation up to 15% and thereafter decreased. Raising the PEG level increased malondialdehyde (MDA) concentration and SOD activity up to 10% PEG and thereafter decreased. However glutathione reductase (GR) and catalase (CAT) activities were decreased in general at the highest levels of PEG. Proliferation of somatic embryos was influenced by their developmental shapes. The proportion of normal embryo developmental shapes were about 50% compared with 20% abnormal shapes at optimum levels of PEG.

Cultivar (cv.) Samani accumulated more organic solutes compared cv. Sewi under normal and stress inducing media. In contrast, lipid peroxidation, GR, SOD and CAT activities were significantly higher in cv. Sewi than in cv. Samani.

These results indicate that the cv. Samani has the ability to tolerate a higher level of drought stress compared to cv. Sewi due to the osmotic re-balancing within its tissues.

Key words: *Phoenix dactylifera*, organic solutes, antioxidant enzymes, embryogenic callus, glycine betaine, proline, polyethylene glycol.

INTRODUCTION

Date palm *(Phoenix dactylifera)* is an economically important commodity crop widely cultivated in arid regions of the Middle East and North Africa. Date palm needs to be vegetatively propagated in order to obtain true to type plants with fruit quality, identical to that of mother plants. *In vitro* micropropagation is increasingly becoming an attractive alternative for commercial large scale propagation of date palm especially for newly reclaimed soils.

In vitro plant regeneration of date palm usually occurs through organogenesis and somatic embryogenesis depending on genotype, explant sources, viability, age of the mother plants, type of the explant used and its physiological status, cultured explant morphogenesis, hormonal manipulation and composition of the culturing media including carbon sources and level used, placement of the explant on the culture media, and several subsequent effects apparent during the *in vitro* process (Helaly and Hanan El-hosieny 2011). Various regeneration protocols, method of multiplication, somaclonal variation in the regenerated population, technical experience, non-synchronous plant production and poor field survival all limit the success of *in vitro* propagation of date palm (Costa Maria and Aloufa 2006**)**. Somatic embryogenesis provides the best method for commercial micro-propagation of date palm however a current limitation is the lack of synchronization of developing somatic embryos (Al-Khayri and Al-Bahrany 2012) especially under stress condition. Tremblay and Tremblay (1995) reported that with black spruce, mannitol was not suitable to study the effect of the osmotic pressure on cell culture regeneration and maturation and polyethylene glycol (PEG) a stress-inducing osmoticum occasionally included in culture medium to stimulate *in vitro* drought stress, was observed to exert modifications on somatic embryogenesis in some plant species (Viji et al. 2012). PEG has been found to influence *in vitro* growth and differentiation in pines (Ishii et al. 2008), in Chestnut (Calic-Dragosavac and Rodosevic 2010**)**; in mango (Mishra et al. 2010) and in tea **(**Suganthi et al. 2012). In relation to date palm tissue culture, the use of PEG as a selection agent for drought stress was demonstrated by El-Sharabasy et al (2008), Helaly and Hanan El-Hosieny (2011) and Al-Khayri and Al-Bahrany (2012). PEG is also considered an important component for the cryoprotection of date palm tissue *in vitro* cultures (Bekheet et al. 2007 and Bekheet 2011). Moreover, a previous studies has shown that supplementation of PEG to the culture medium of date palm reduced hyper hydration and enhanced maturation and germination of somatic embryo (Al-Khateeb 2008). The incorporation of PEG to date palm cultures has also been shown to elicit increased accumulation of proline an indicator of osmotic stress (Al-Khayri and Al-Bahrany 2004a). It has been demonstrated that environmental osmotic stress leads to major alterations in carbohydrate metabolism, lipid peroxidation and/or an induction of oxidative stress in date palm plant tissues (Helaly and Hanan El-Hosieny 2011). Lipid peroxidation requires active O_2^- uptake and involves the production of the superoxide radical; O_2^- (Hatung, 2004 and Zhaleh et al. 2013). Other highly reactive oxidative chemical species are singlet oxygen (O_2^-) hydroxyl free radical (OH^-) and hydrogen peroxide (H_2O_2) all of which initiate lipid peroxidation (Bor et al. 2002). Constitutive and/or induced activity of superoxide dismutase (SOD) and other antioxidants such as peroxidase (POX), ascorbate peroxidase (APOX), catalase (CAT) and glutathione reductase (GR) is essential under oxidative stress conditions. Alterations in the sugar signaling pathways interacting with stress pathways to modulate metabolism have also been recorded (Gupta and Kaur, 2005 and Mirzaee et al. 2013).

In date palm several works have been published describing culture media and optimised protocols for organogenesis or somatic embryogenesis (Al-Khateeb 2008; Helaly and Hanan El-Hosieny 2011). Although numerous factors relevant to date palm somatic embryo genesis have been investigated (Al-Khayri 2013), studies related to the role of PEG on synchronization of somatic embryo development and their physiological response have not been reported and it is not understood. Little information is available concerning the effects of increasing osmotic stress in the culturing media on physiological parameters related to lipid peroxidation and antioxidative enzymes during the embryogenic cell suspension culture. Therefore, the objective of the present investigation was to determine the effects of stress caused by increasing exogenous supply of PEG on growth and synchronization of embryo development in two genotypes of date palm cell suspension grown in Egypt. Lipid peroxidation, organic solutes and anti-oxidative enzyme changes during the embryogenic cell suspension culture were also examined.

MATERIALS AND METHODS

The present investigation was carried out at the laboratories of plant tissue culture Department of Genetic Engineering and Biotechnology Research Institute, GEBRI, Sadat city, Menofia University, Egypt during the period from June 2013 to October, 2014. Chemical analyses were carried out at the central Laboratories of the Department of Agricultural Botany, Faculty of Agriculture, Mansoura University, Egypt.

Plant material:

Shoot tip explants, about 8 cm long, from two genotypes of date palm, cv. Sewi, belonging to the semi-dried type and cv. Samani a soft dessert type, were excised from female suckers of 3 year old mother plants. The two genotypes were provided by the Horticultural Research Institute, ARC, Egypt. The shoot tip explants and the surrounding leaf primordia were placed in a chilled antioxidant mixture containing 150 mg L^{-1} of ascorbic and citric acid to prevent browning (Helaly and Hanan El-Hosieny 2011). They were surface sterilized for 20 min with

2% Desogerm, followed by 20 min immersion in 1.5% w/v commercial sodium hypochlorite (30% v/v Clorox commercial bleach) and 0.1 mL per 100 mL of Tween 20 as a disinfection solution as described by Helaly and Hanan El-Hosieny (2011).

Embryogenic callus, induction and multiplication:

Callus induction was conducted according to El-Hadrami et al. (1998) with modifications as follows: the explants were cultured on callogenesis induction medium containing MS media (Murashige and Skoog 1962) supplemented with vitamins, 30 gL^{-1} sucrose, 150 mg L^{-1} activated charcoal; (A.C, acid-washed neutralized,) 6.8g L^{-1} carrageenan, 5 mg L^{-1} 6-benzylamino-purine (BAP), 5 mg L^{-1} of dichlorophenoxy acetic acid (2,4-D). Incubation was in complete darkness at 25±2 ºC. After 6 months, the friable calli formed were selected and transferred to proliferation fresh media containing 0.1 mg L^{-1} of BAP and 0.5 mg L^{-1} of 2, 4-D. Tissues were incubated suspension again to 12 week at 25±2 ºC in the dark and sub-cultured to freshly medium every 4 weeks (3 subcultures). All media were adjusted to pH 5.7 with 1 N KOH, dispensed in test tubes (15 mL per tube) or GA-7 Magenta vessels (50 mL per vessel) and autoclaved for 15 min at 121 ºC and 1.1 kg cm^{-2}. All chemicals were obtained from Sigma Aldridge Chemical Co., St. Louis, MO, USA.

Establishment of cellular suspension and PEG treatment:

In accordance with the cell method described by Côte et al. (2000) the obtained embryogenic callus explants (500 mg) were cut with a sterile scalped into as small pieces as possible and then transferred to 50 mL of liquid MS basal medium in 250 mL Erlenmeyer flasks. The contents of the Erlenmeyers was filtered using a sieve (500 µm diameter) and the obtained filtrate incubated on a rotary shaker (100 rpm) at 25 ºC under conditions of 16 h photoperiod (irradiance of 50 µmol. m^{-2} sec^{-1}) provided by cool-white fluorescence lamps for 10 days. The liquid culture medium was half strength MS basal media supplemented with 2.4-D (0.1 mg L^{-1}), BAP (0.5 mg L^{-1}), sucrose (30 g L^{-1}) and polyethylene glycol-6000 (PEG) as an osmotic agent. The PEG was added to the media at different levels denoted as 0(control), 5, 10, 15, 20 % w/v). Growth of the embryonic cell suspension was recorded for each genotype after 8 weeks culture after supplementation with PEG. To assess the effect of PEG on culture growth, the resultant somatic embryo numbers were counted. In addition certain biochemical constituents were estimated. The resultant embryos were sub-cultured on hormone-free solid MS media under the same light conditions at 25±2ºC to examine the consequent effects of PEG on regeneration.

Estimation of lipid peroxidation:

Lipid peroxidation was determined according to Madhava Roa and Strecty (2000) by estimating the malondialdehyde (MDA) concentration in 500 mg fresh embryo cell suspension (ECS). MDA is product of lipid peroxidation by thiobarbituric acid reaction. The MDA concentration was calculated at 532 nm absorbance using extinction coefficient of 1555 mM^{-1} cm^{-1}. Correction was carried out by subtracting the absorbance at 600 nm for non-specific turbidity.

Protein and antioxidant enzymes extraction:

500 mg from the ECS were frozen in liquid N and stored at -20 ºC until enzyme assays. The total soluble protein (TSP), hydrogen peroxide (H_2O_2) and antioxidant enzymes were extracted according to the methods described by Lecouteux et al. (1993). Samples (250 mg) were homogenized and extracted with 3 mL of 0.25 M Na phosphate buffer (pH 7.8) supplemented with 1 mM ethylene diamine tetra acetic acid (EDTA) and 2 % (w/v) polyvinylpyrrolidone polymer (PVPP). The homogenate was centrifuged at 13000 G for 40 minutes. The supernatant was used as the crude protein extraction for enzyme activity and protein content assays. All assays were done at 4 ºC.

Estimations of free amino acids and phenolic compounds:

Total soluble protein contents of the enzyme extracts was determined spectrophotometrically (Shimadzn UV-1600) at 595 nm according to the method described by Bradford (1976) using bovine serum albumin; BSA as a standard. Total free amino acids was determined according to Duby and Rani (1989a,b) using 0.1 for the extract and 5 ml ninhydrin reagent, shaken vigorously, heated for 10 minutes in a boiling water bath, cooling and spectrophotometrically recorded at absorbance 570 nm.

Total sugars were determined by phenol-sulphoric acid method as described by Sadasivam and Manickam (1996).

Proline was determined by the modified ninhydrin method of Magne and Larher (1992) using 2 mL of the previous supernatant, 2 mL of acid ninhydrin and 2 mL of glacial acetic acid, then boiling for 60 minutes. The mixture was treated with toluene and free proline was quantified spectrophotometrically at an absorbance of 520 nm from the organic phase. A calibration curve was made with L-proline as a standard. Total phenolic compounds were determined as described by Singleton and Rossl (1965) using Folin-Ciocaltean reagent and calculated as mg catechol per 100 g F.Wt.

Estimation of glycine betaine and organic acids:

Glycine betaine (GB) was determined spectrophotometriccally at 365 nm in the extract according to the method of Greive and Grattan (1983). Total water soluble organic acids (TOA) extraction was performed as described by Huang and Redmann (1995) using water:methanol/chloroform (2:1) water and chloroform in the ratio of 1.1:3.5:1.2:1.2. The extract, after 12 h, was filtered and the supernatant was aspirated in covered vials and determined by titration with 0.005 N NaOH using 0.04% aqueous bromathymol blue as an indicator which became green at pH 7.0.

Estimation of hydrogen peroxide and antioxidant enzymes activities:

Hydrogen peroxide concentration was estimated according to the procedure of Velikova et al. (2000) using 0.5 mL of the previous supernatant which was added to 0.5 mL of 10 mM K-P buffer at pH7.0 and 1mL of 1M KI. The absorbance of the mixture was measured at 390 nm. The concentration of H_2O_2 was determined using a standard curve.

Superoxide dismutase (EC1.15.1.1) (SOD) activity was assayed based on the method of Beauchamp and Fridovich (1971) which measures the inhibition in the photochemical reduction of nitroblue tetrazolium (NPT) spectrophotometerically at 560 nm. One unit of enzyme activity was defined as the quantity of SOD required to produce a 50% inhibition of reduction of nitroblue-tetrazolium (NBT) and the specific enzyme activity was expressed as unit's mg^{-1} protein g F.Wt. The reaction mixture contained 50 mM Na phosphate buffer (pH 7.8), 33 μM NBT, 10 mM L-Methionine, 0.66 mM EDTA and 0.0033 mM Riboflavin. Reactions were carried out at 25 ºC, under a light intensity of about 300 $\mu Mol^{-1} m^{-1} s^{-1}$ through 10 min.

Peroxidase (EC1.11.1.7) activity (POX) was assayed based upon the method described by Herzog and Fahimi (1973) which measures the increase in absorbance at 465 nm, by the rate of formation of 0.15 M Na phosphate citrate buffer the oxidized diaminobenzidine-tetrahydrochloride dehydrate (DAB). The reaction mixture contained DAB solution (dissolved gelatin solution and contained 50% (w/v), and 0.6% H_2O_2). The increase in A_{465} was followed for 3 min. One enzyme unit is defined as μmol mL^{-1} destroyed H_2O_2 per min.

Catalase (EC 1.11.1.6) activity (CAT) was determined according to Bergmeyer (1970) which measures the decline of the extinction of H_2O_2 at the maximum absorption at 240 nm. The reaction mixture contained 0.05 M Na phosphate buffer (pH 7.0) with 1 mM EDTA and H_2O_2 (3%). The decrease in the absorption was followed for 3 min and μmol H_2O_2 destroyed per min was defined as one unit CAT. Glutathione reductase (EC.1.6.4.2) (GR) activity was measured according to Foyer and Halliwell (1976) which depends on the rate of decrease in the absorbance of oxidized glutathione (GSSG) at 340 nm. The reaction mixture contained 25 mM Na-phosphate buffer (pH 7.8), 5 mM GSSG, 1.2 mM $NADPHNa_4$. The reaction was carried out for 3 min and activity of GR was calculated from the reduced GSSG concentration by using the extinction coefficient 6.2 mM^{-1} cm^{-1}. One enzyme unit is defined as μmol mL^{-1} oxidized GSSG per min.

Ascorbate peroxidase (EC.1.11.1.11) (APOX) activity was assayed according to Nakano and Asada (1981) which depends on the decrease in absorbance at 290 nm as ascorbate is oxidized (extinction coefficient of 2.8 mM^{-1} cm^{-1}). The reaction mixture contains 50 mM Na-phosphate buffer (pH 7.0), 0.5 mM Ascorbate, 0.1 mM EDTA Na2 and 1.2 mM H_2O_2. One enzyme unit is defined as μmol mL^{-1} oxidized ascorbate per min.

Experimental design and Statistical data Analysis:

A completely randomized block design with 6 replicates was used in these experiments. The data obtained from two independent experiments were subjected to analysis of variance (ANOVA). The mean differences were compared by least significant difference, LSD ($p<0.05$) (Steel and Torrie 1980).

RESULTS AND DISCUSSION

1: Growth of the embryogenic callus:

The degree of embryogenic callus (EC) formation and its fresh weight as well as the percentages of normal embryos callus shapes were increased whereas abnormal embryogenic callus was decreased with the supplementation of PEG to MS medium up to 15% (Table 1). However increasing PEG level more than 10% recorded less increase in callus formation and its fresh weight in both genotypes. Moreover, PEG at both of the highest levels (15 and 20%) decreased the degree of normal EC shape and increased abnormally shaped ones. These results were the same for both cultivars Sewi and Samani, in all treatments used in comparison with the controls. The best treatment was found with MS + 10% PEG followed by MS + 15% PEG for both genotypes. The normal embryogenic callus shapes obtained in all treatments were yellowish-white in colour, aggregated but friable and composed of minute nodules.

Abnormal EC shapes were partially dependent on the genotype and the treatments used.

The genotype Samani showed lower values than Sewi regarding the degree of EC formation as well as the percentage of normal shape and callus fresh weight. Similar results were reported by Suganthi et al. (2012) on tea, Ali et al. (2010) on walnut and Al-Ka'aby and Luma, (2011) on date palm. However, there were differences between these reports regarding the optimum level of PEG. Sané Djibril et al. (2005) working on date palm found that, PEG is the preferred carbohydrate for induction, proliferation and embryo maturation.

Table 1: Effect of polyethylene glycol (PEG) on the degree of embryogenic callus (EC) induction and its fresh weight as well as the percentages of normal and abnormal embryogenic callus shapes of two date palm genotypes after two months from PEG supplementation.

Characters	Genotype	PEG-6000 levels %				
		0(Control)	5%	10%	15%	20%
Degree of EC induction	Samani	+	+++	+++	++	+
	Sewi	+	++	+++	++	+
*EC mass g FWt per flask	Samani	7.15±0.3	14.6±0.01	15.7±0.2	12.3±0.16	5.30
	Sewi	6.70±0.16	10.7±0.3	13.3±0.4	9.4±0.11	6.11
Normal EC. Shape%	Samani	60	65	70	45	30
	Sewi	55	62	65	40	25
Abnormal EC. Shape%	Samani	40	35	30	55	70
	Sewi	54	38	35	60	75

*Values are means ± standard error of six replicates from two experiments.

Degree of embryogenic callus (EC) as **Pottino (1981)**

2: Accumulation of organic solutes

Total soluble protein (TSP), proline, glycine betaine (GB), total soluble phenol (TSPh), total sugars (TS) and total soluble organic acids (TOA) were all increased due to the supplementation of PEG to the MS basal media and the increase was concentration dependent (Table 2).

Cultivar Samani accumulated more organic solutes compared with cv. Sewi under normal and/or stressed media indicating that cv. Samani has a greater ability to tolerate drought stress caused by PEG compared to cv. Sewi. Bartels and Sunkar (2005) also found a strong correlation between osmotic stress tolerance and sugar accumulation. Gupta and Kaur (2005) suggested that plants have two systems for hexose sensing: namely hexokinase-dependent (HxK) and the HxK-independent pathway. The HxK-independent system requires the phosphorylation of sugars while the dependent one senses sugars as such (Smeekens 2000) and sugars can act as regulatory signals that control the expression of various genes involved in many processes. The evidence in the favor of HxK-dependent signaling came from the observations that those sugars analogues that can be phosphorylated by HxK were able to trigger repression of photosynthetic genes (Jang and Sheen, 1994 and Mahmood 2013). Furthermore, metabolism of sugars phosphates was not necessary to cause repression because 2-deoxy glucose; 2-DG and 2-deoxy mannose that can not be metabolized after phosphorylation could also cause severe repression. These findings suggested that sugar signaling pathways do not overlap with downstream glucose metabolic pathways (Gupta and Kaur 2005).

The results also indicate that the increase in osmotic solute accumulation, especially proline and total sugars, seems to be related to PEG stress in date palm, not as a consequence of their tissue reaction to stress damage. The accumulation of organic solutes in the stressed plants may play an important role as an osmoregulation and/or as an osmoprotectant of date palm callus against stresses. It has been reported that free amino acids (AA) and proline accumulation may contribute osmotic adjustment at the cellular level (Tripathi et al. 2007). A direct consequence of higher osmolyte accumulation in tolerant cultivars of wheat is the maintenance of comparatively higher relative water content (RWC) (Misra and Saxena, 2009) and up-regulation of specific enzymes of proline metabolism (Misra and Gupta, 2006).

The accumulation of total free amino acids (TAA) in stressed tissues noticed in the present investigation on PEG-containing media may be due to inhibition of protein syntheses and/or enhancement of protein degradation to providing AAs needed for new protein syntheses required for growth or survival on the modified media (Yadav et al. 1999, EL-Beltagi et al. 2013) in addition to withstanding the other negative effects induced by stress inducing media. The increases in total soluble protein caused by PEG supplementation (Table 2) may be due to an activated synthesis of adaptive protein under stress conditions. Moreover, the differences noticed between the treated genotypes of date palm seem to be associated with the ability of plant tissues to survive the severe condition. The possibility that plants can from specific protein types for adaptation to high stress condition has also been reported previously (Al-Mulla et al. 2013) and the role of protein in the adaptation response to stress has been also reported (Hatung, 2004). Sperling et al. (2014) added that, specific substances are formed in the plant tissues which protect the plasma colloids from coagulation caused by the electrolytes effect, and such substances may be hydrophilic proteins. The hormonal modification of protein metabolism due to stress has also been reported elsewhere (Mona Dawood et al. 2012).

Proline accumulation was also noted in the PEG stressed date palm tissues (Table 2) and it was reported by Misra and Saxena (2009) that proline has multiple functions such as osmotic pressure regulation, protection of membrane integrity, stabilization of enzymes proteins, maintain appropriate $NADP^+$/ $NADPH+H^+$ ratio, and scavenger of the free radicals. Jain et al. (2001) added that, proline can be considered a major source of energy and nitrogen during immediate post-stress (recovery) metabolism. Therefore, the accumulation of proline apparently supplies energy for growth and survival thereby contributing to stress tolerance. Alquarainy (2007) reported that over accumulation of proline in stressed plants may be due to the strategies adapted by the plants to cope up with the stress condition. It was found that, the accumulation of proline may be associated with the increase in synthesis of Δ pyrroline carboxylate synthetase (P5(S) and P5CS mRNA (Verslues and Sharma, 2010; Xia et al. 2014) and pyrroline 5 carboxylate reductase (P5CR) (Misra and Gupta 2006) as well as ɣ-glutamy kinase activity (Misra and Saxena, 2009) or the low activity of degrading enzyme, proline oxidase (EC.1.5.99.8), localized in inner mitochondrial membrane (Misra and Saxena, 2009) and cytoplasmic proline dehydrogenase (EC.1.5.1.2) (Delanay and Verma, 1993 and Al-Zubaydi et al. 2012) to negligible rate.

The accumulation of GB and TSPh in stressed date palm especially at the high levels of PEG supplementation (Table 2) may be due to their important as a compatible solutes. Sakamoto and Murata (2002) reported that GB interacted with both the hydrophobic and hydrophilic domains of macromolecules without perturbing cellular functions maintaining an osmotic balance between the intracellular and extracellular environments and by stabilizing the structures of complex proteins like antioxidants enzymes, as well as bio membranes and other functional units such as the oxygen-involving photosystem Π complex (Rhodes and Hanson, 1993). Alquarainy (2007**)** stated that application of AsA increased GB content in bean and pea seedlings growth under salt stress. This finding may be due to the effectiveness of AsA on increasing glycine betaine formation by stimulation of its biosynthesis. Similarly, Helaly and Hanan El-Hosieny (2011) found that GB was accumulated and contributed to the maintenance of organic acids in antioxidant treated wheat plants under normal and salinized condition.

The increasing effects of PEG supplementation on total soluble phenols (Table 2) may be due to its effects on reducing oxidation of the phenollic compounds and its correlation with high level of AsA. Ichihashi and Kako (1977) and El Dawayati et al. (2012**)** found that AsA did not prevent phenol oxidation but prevented quinone polymerization reducing the probability to negatively react with proteins. Sané Djibril et al. (2005) found that phenolic exudate related compounds were decreased in explants of date palm after 24 h in media containing high levels of ascorbate and citrate.

The increase in organic acids due to the increase in PEG levels noticed in both genotypes of date palm as shown in the present investigation (Table 2) gives an indication about the activities of the mitochondria and cytosol respiration, as well as the equilibration of any cation excess counteracting the catalytic mechanism of passive water absorption. Several investigations have recorded a positive correlation between organic acid accumulation (citric, malate) all linked to oxidation and stress tolerance (Bourgeais-Chailcou and Guerrier, 1992 and Suriyan et al. 2013). The actual role of organic acid accumulation in the stressed plants may be attributed to their osmotic activity in the plant tissues.

The correlation between the accumulation of total sugars and proline, as organic solutes, noticed in the present investigation (Table 2) due to increasing PEG supplementation, may be due to the importance of increasing proline synthesis and/or inhibiting the enzymes involved in the degradation of proline. Hare and Cress (1996) and Amirjani (2010) found that, m-RNA transcript encoding P5CP was increased in phloem tissue in response to water deprivation. The dramatic increase in transcription of the gene may be related to the finding of Heineke et al. (1992) and Verslues and Sharma (2010**)** who found, on potato, that when sucrose phloem loading was blocked, proline was accumulated at a high level. Ma et al. (2004) reported that, GB, as an osmoprotectant, induced the accumulation of sugars and free proline as organic solutes and these compounds play an important role in increasing the osmotic pressure of the cytoplasm (Kholova et al. 2009). Tajdoost et al. (2007) added that these organic molecules act as osmolytes and play a role in osmotic adjustment in non-halophytes. Bartels and Sunkar (2005) found a strong correlation between sugar accumulation and osmotic tolerance. They added that sugars as osmolytes enable plants to maintain better water relations under stress conditions. The current hypothesis is that sugars act as osmolytes and/or protect specific macromolecules and contribute to the stabilization of membrane structures. In this context, Schnapp et al. (1990) and Sperling et al. (2014) reported that sugar accumulation is the result of an enhanced efficiency in the use of carbon coupled to a reduction in cellular metabolism that could favour the accumulation of respiratory substrate to support the osmotic adjustment required to survive in stress media.

It could be concluded that, sucrose and other sugars have dual roles in plant metabolism. They are involved in various metabolic events and also regulate various genes especially those involved in photosynthesis, sucrose metabolism and synthesis of osmoprotectants, which act as protectants against abiotic stresses.

Table 2: Concentrations (mg/g F.Wt) of organic solutes, soluble protein (TSP), total amino acids (TAA), proline, glycine betaine (GB), total soluble phenols (TSPh), total sugars (TS) and total water soluble organic acids (TOA) during embryogenic cell suspension culture of date palm genotypes as affected by PEG supplementation.

PEG-6000 Levels %	Genotypes	TSP mg g^{-1} F.W	TAA mg g^{-1} F.Wt	Proline mg g^{-1} F.Wt	GB mg g^{-1} F.Wt	TSPh mg catechol/100g F.W	TS mg g^{-1} F.Wt	TOA mg g^{-1} F.Wt
0 (Control)	Samani	13.4	85.86	11.64	1.57	12.4	20.25	40.5
	Sewi	11.6	78.34	9.82	1.25	14.6	19.86	46.8
	Mean	12.5	82.1	10.73	1.41	13.5	20.06	43.65
5 %	Samani	14.5	90.25	17.54	4.08	18.8	28.0	52.8
	Sewi	13.8	82.57	15.46	3.68	19.4	29.54	55.2
	Mean	14.15	86.41	16.5	3.88	19.1	28.77	54.0
10 %	Samani	16.2	102.8	20.48	5.94	22.5	32.35	60.6
	Sewi	14.8	94.5	19.22	5.12	20.4	32.68	68.4
	Mean	15.5	98.65	19.85	5.53	21.45	32.52	64.5
15 %	Samani	14.2	87.62	18.24	5.66	18.2	31.48	51.4
	Sewi	13.4	80.12	16.86	4.24	17.0	31.96	50.6
	Mean	13.8	83.87	17.55	4.95	17.6	31.72	51.0
20 %	Samani	12.4	76.94	13.12	1.34	12.8	21.28	41.2
	Sewi	11.5	64.82	10.64	1.15	13.6	20.46	45.8
	Mean	11.95	70.88	11.88	1.245	13.2	20.87	43.5
Mean	Samani	14.14	88.70	16.24	3.72	16.94	26.67	49.3
	Sewi	13.02	80.07	14.4	3.09	17.0	26.93	53.36
LSD. at 5 % for								
Genotypes (G)		0.11	0.76	0.13	0.07	0.21	0.40	1.7
PEG-6000 levels (L)		0.17	0.81	0.15	0.09	0.33	0.53	2.0
G x L		0.23	1.63	0.33	0.13	0.99	1.33	4.6

3 – Lipid peroxidation; MDA content

Data show that, lipid peroxidation was significantly higher in Sewi than in Samani under the control conditions (zero PEG level) as well as under higher levels of PEG (Table 3). The rate of lipid peroxidation increase was recorded to be higher from zero level to 10% than from 10 to 15 or 20% in both genotypes. However, increasing PEG level increased MDA concentration significantly up to 15% and thereafter tended to decrease. The lowest values were recorded at 20%. In contrast, it was found that increasing PEG levels increased MDA concentration of Sewi more than Samani. These results supported the observation above that the Samani genotype has a higher resistance capability under PEG stress (Table 3). A higher degree of lipid peroxidation was noticed in Sewi compared to the tolerant genotype Samani. Cell suspension culture may result in an increase in membrane permeability or loss of membrane integrity leading to an increase in solute leakage, hence decreasing resistance to stress (Bor et al. 2002). This protection might be a result of by significantly higher constitutive activities of SOD and induced activities of POX, APOX, CAT, and GR in the Samani genotype. It has been demonstrated by Sané Djibril et al. (2005) that stress conditions increased lipid peroxidation or induce oxidative stress in plant tissues and resulted in a high degree of membrane deterioration. They added that, lipid peroxidation, which can be initiated by ROS, severely affects functionality and integrity of cell membranes. It requires active O^{-}_{2} uptake and involves the production of the superoxide radical (O^{-}_{2}). The other highly reactive chemical species all involve singlet oxygen (O^{-}_{2}) and include the hydroxyl free radical (OH^{-}) and hydrogen peroxide (H_2O_2) all of which initiate lipid peroxidation (Dhindsa et al. 1981 and Filippou et al. 2014 and Talbi et al. 2015). Constitutive and/or induced activity of SOD and other antioxidants such as POX, APOX, CAT and GR is essential if such ROS are to be counteracted. According to Seckin et al. (2009) MDA has been frequently described as a suitable biomarker for lipid peroxidation under stress condition.

4- Hydrogen peroxide; H_2O_2 and antioxidant enzymes activities

4-1 H_2O_2 and SOD

Superoxide dismutase (SOD) activity which catalyzes the conversion of the superoxide anion to H_2O_2 was increased due to the increase in PEG level in both genotypes (Table 3). The rate of increase in SOD activity was higher in cv. Sewi than cv. Samani at 5, 10, and 15 % PEG levels but at 20%, SOD activity was decreased. In the control (zero PEG) SOD activity in cv. Samani was increased significantly whereas it stayed almost the same in cv. Sewi during the embryogenic cell suspension indicating that Samani has a high constitutive level of SOD activity. This may enabled cv. Samani to resist the potential oxidative damage without the requirement to increase the SOD activity further. In addition, cv. Samani has a higher dis-mutating capacity under 5, 10 and 15% PEG level when compared with the absolute enzymatic values of the control. In this context, Acar et al. (2001) found a higher constitutive and induced level of SOD in more tolerant cotton and barley cultivars under drought stress. Similar results were reported by Helaly and Hanan El-Hosieny (2011) on date palm.

The increase in SOD activity, noticed in the present investigation due to increasing PEG level, was supported by the finding of Bohnert and Jensen (1996) and Helaly and Hanan El-Hosieny (2011) on sweet orange who reported that drought treatment enhanced SOD activity by increasing H_2O_2. Similar results were reported by Subbarao (1999) on tobacco cell culture.

4-2. POX and APOX

POX activity, which decomposed the H_2O_2 produced by SOD changed with response to both genotype and PEG levels (Table 3). Unlike SOD, POX activity was increased remarkably with increasing PEG level in the cv. Samani and in cv. Sewi there was also an increase in POX activity up to 15% of PEG which then decreased at 20%. The control activity of POX did not change significantly in cv. Sewi while it increased slightly in cv. Samani.

The activity of APOX, which also decomposes H_2O_2 was increased in both genotypes with an increase in PEG level (Table 3) during the embryogenic cell suspension culture. However, stress caused by an increase in PEG level induced APOX activity which was significantly higher in cv. Samani than in cv. Sewi after incubation at 5, 10 and 15% PEG level. These data can be considered as an indication that cv. Samani has a higher capacity to decompose H_2O_2 more rapidly compared with cv. Sewi.

The data also indicated that the contributory role of APOX to decompose H_2O_2 in cv. Samani is higher (Table 3). In addition, there was an association between the increase noted with SOD and APOX activities in the cv. Samani genotype. These results suggested that SOD and APOX are working more efficiently to decompose oxidants such as O^-_2 and H_2O_2 which might possibly be produced during stress conditions caused by increasing the PEG supplementation level. In addition these results suggested that H_2O_2 in cv. Samani during the tissue culture technique (TCT) is more efficiency eliminated by ascorbate-glutathione cycle in which APOX acts a strong catalyst together with MDHAR, DHAR and GR to decompose H_2O_2 more rapidly similarly to that found by Bor et al. (2002) on Beta sp. Since, POX is among the enzymes that scavenges H_2O_2 in chloroplasts which is produced through dis-mutation of O^-_2 catalyzed by SOD (Asada and Takahashi 1987 and Kamrun et al. 2015), the increased in POX activity was expected in both genotypes under stress condition. These increases may be attributed to an increase in the activity of POX encoding genes and/or an increase in the activation of already enzymes (Dionisio, Sese and Tobita (1998). APOX uses ascorbate as the electron donor for the reduction of H_2O_2 and is well known to be important in the detoxification of H_2O_2 (Asada and Takahashi 1987 and Filippou 2014). Wang et al. (1999) showed that over expression of APOX gene in plants increases protection against oxidative stress.

4-3. CAT and GR

Catalase (CAT) activity, another scavenger of H_2O_2, was increased in both genotypes Sewi and Samani under the effects of 5, 10 and 15% PEG levels and thereafter decreased (Table 3). Genotype Samani was again higher at 5, 10 and 15 %CAT activity in control groups in both genotypes (PEG-free media) was higher and significantly increased s during embryogenic cell suspension culture. It could be considered a response to stress induced oxidative damage suggesting enzymatic removal of H_2O_2 by CAT (Shalata and Neumann 2001).

GR activity another enzyme in Asada-Halliwell pathway, was increased significantly due to an increase in PEG level in both genotypes Sewi and Samani (Table 3). However, the rate of increase was less than that of CAT activity. At 5, 10 and 15% PEG level, the induction of GR activity, was higher in Sewi than in Samani genotype of date palm. Moreover, GR activity in the control was high and increased significantly only in Samani. In the studies of Foyer and Halliwell (1976) on *Arabidopsis* and Helaly and Hanan El-Hosieny (2011) on date palm SOD, APOX and GR activities have been reported to increase during drought stress. Ünyayar (2004) attributed the increase in GR activity, under drought stress, to the increase in APX activity which would increase the demand for ascorbate generation mediated through increased GR activity.

The results obtained in this study conclude that increasing PEG level in embryogenic callus media can vary the activation of the antioxidant enzymes. The data suggested that the Samani genotype was more tolerant and this tolerance was upregulated by increasing osmotic stress induced by increasing PEG levelsOsmotic stress effects may be lead to changes in oxygen free radical levels which in turn lead to induction of the antioxidant defense system (Shalata and Neumann 2001). Enzymatic activities of the Halliwell-Asada pathway have been separately associated with different stresses situations and the balance between the formation and detoxification of AOS is critical to cell survival during periods of abiotic stress (Foyer and Halliwell 1976).The important components of protective systems are enzymatic defenses such as SOD, CAT, APX and GR which savage O^-_2, H_2O_2 and OH (Wang et al. 2014) and are predominantly responsible for controlling free radicals and consequently the ability for growth and development as shown in the present investigation.

Table 3: Lipid peroxidation; MDA: (Malondialdehyde nmol g^{-1} F.Wt), hydrogen peroxide; H_2O_2(µM g^{-1} F.Wt) and the activities of antioxidant enzymes (unit g^{-1}F.Wt) examined during the embryogenic cell suspension culture of two date palm genotypes as affected by PEG supplementation levels.

PEG-6000 Levels %	Genotypes	MDA nmol g^{-1} F.Wt	H_2O_2 µM g^{-1} F.Wt	SOD unit g^{-1} F. Wt	POX unit g^{-1} F. Wt	APOX unit g^{-1} F. Wt	CAT unit g^{-1} F. Wt	GR unit g^{-1} F. Wt
0 (Control)	Samani	500	13.64	30.6	25.5	4.50	8.22	50.3
	Sewi	450	12.82	28.9	28.8	5.24	8.54	46.3
	Mean	475	13.23	29.75	27.15	4.87	8.38	48.3
5 %	Samani	700	18.54	50.3	20.2	3.65	6.47	65.2
	Sewi	640	17.46	48.0	24.4	4.34	7.28	60.0
	Mean	670	18.33	49.0	22.3	3.95	6.82	62.5
10 %	Samani	900	22.48	54.5	19.7	2.60	5.45	85.4
	Sewi	830	21.22	52.6	21.6	2.82	6.86	78.5
	Mean	865	21.85	53.7	20.0	2.70	6.27	81.5
15 %	Samani	620	19.24	48.4	20.2	3.45	7.25	68.6
	Sewi	600	19.86	45.2	22.3	3.26	8.68	62.8
	Mean	610	19.55	46.5	21.6	3.35	7.92	65.1
20 %	Samani	520	12.86	32.5	24.6	4.53	7.87	48.0
	Sewi	460	11.24	27.4	28.1	5.40	7.94	46.9
	Mean	490	12.05	29.5	26.3	4.95	7.35	47.4
Mean	Samani	648	17.35	43.26	21.04	3.74	7.05	63.5
	Sewi	596	16.52	40.42	25.04	4.21	7.86	58.9
LSD. at 5 % for								
Genotypes (G)		0.16	0.64	0.37	0.17	0.28	0.20	1.27
PEG-6000 levels (L)		0.26	0.73	0.35	0.19	1.21	0.67	2.16
G x L		0.28	1.43	0.42	0.13	1.96	1.83	4.57

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Key Message

This paper reports on the somatic embryogenesis of date palm under PEG-induced osmotic stress. It shows that increasing stress increased the proportion of embryos with normal morphology. Increasing PEG stress also increased the levels of antioxidant enzymes and compatible solutes. There were clear differences between cultivars tested suggesting that there is genetic variation in date palm worthy of exploitation for drought stress resistance