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ARTÍCULO DE INVESTIGACIÓN

IMPACT OF FOLIAR APPLICATION OF ASCORBIC ACID AND α-TOCOPHEROL ON ANTIOXIDANT ACTIVITY AND SOME BIOCHEMICAL ASPECTS OF FLAX CULTIVARS UNDER SALINITY STRESS

Impacto de las aplicaciones foliares de ácido ascórbico y α- tocoferol en la actividad antioxidante y algunos aspectos bioquímicos de cultivares de lino sometidos a estrés por salinidad.

Hala M.S. EL-BASSIOUNY[1], Mervat Sh. SADAK[1]

[1] Botany Department, National Research Centre, 33 Al Behoos Street, Dokki, Cairo, Egypt

For correspondence. mervat_sh24@yahoo.com

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ABSTRACT

The interactive effects of saline water (2000, 4000 and 6000 mg/l) and foliar application of 400 mg/l of ascorbic acid (Asc) or α–tocopherol (α-Toco) on three flax cultivars (Sakha 3, Giza 8 and Ariane) were conducted during two successive seasons 2011/2012 and 2012/2013. The results showed that total soluble carbohydrates, free amino acids and proline contents were significantly increased with increasing salinity levels in the all three tested cultivars except free amino acid content of Giza 8 which showed insignificant decrease. While, nucleic acids (DNA and RNA) showed significant decreases compared with the corresponding control. Moreover, applications of vitamins (Asc or α-Toco) as foliar spraying increased all mentioned contents compared to the corresponding salinity levels. On the other hand, lipid peroxidation and activity levels of polyphenol oxidase, peroxidase and catalase enzymes showed significant increases with increasing salinity levels of all tested three cultivars, while the behaviour of superoxide dismutase activity showed an opposite response as compared with the control in Sakha 3 and Giza 8. Treatments with Asc or α-Toco induced significant reduction in lipid peroxidation and activities of polyphenol oxidase, peroxidase of the all three tested cultivars. Meanwhile, superoxide dismutase increased in all three cultivars, and catalase activities increased only in Sakha 3 cultivar under salt stress as compared with reference controls. Some modifications are observed in protein patterns hence some proteins were disappeared, while certain other proteins were selectively increased and synthesised of a new set of proteins were induced, some of these responses were observed under treatments and salinity, while others were induced by either treatments or salinity.

Keywords: antioxidant enzymes, flax, lipid peroxidation, protein profile, salinity, α-Toco.

RESUMEN

La interacción de los efectos del agua salada (2000, 4000 and 6000 mg/l) y la aplicación foliar de 400 mg/l de ácido ascórbico (Asc) o α–tocopherol (α-Toco) en tres cultivares de lino (Sakha 3, Giza 8 y Ariane) se analizó durante dos estaciones sucesivas (2011-2012). Los resultados mostraron que los contenidos de carbohidratos solubles totales, aminoácidos libres y prolina aumentaron significativamente con los niveles crecientes de salinidad en los tres cultivares probados, excepto en el cultivar Giza 8, en el cual el contenido de aminoácidos libres se redujo. Al mismo tiempo, los ácidos nucleicos (ADN y ARN) mostraron disminuciones significativas en comparación con los controles correspondientes. Mientras que aplicaciones foliares de vitaminas (Asc o α-Toco) incrementaron los contenidos mencionados en comparación con los niveles de salinidad. De otro lado, la peroxidación de lípidos y la actividad de la polifenol oxidasa, la peroxidasa, y la catalasa mostraron incrementos significativos con los niveles crecientes de salinidad en

los tres cultivares analizados. Mientras tanto, la actividad superóxido dismutasa se incrementó en los tres cultivares y la actividad catalasa se incrementó únicamente en el cultivar Sakha 3 bajo estrés salino, en comparación con los controles. Se observaron algunas modificaciones en los patrones de proteínas. Así, algunas proteínas desaparecieron mientras que otras incrementaron e incluso un nuevo set de proteínas fue inducido. Algunas de estas respuestas fueron observadas en los tratamientos y en la salinidad, mientras que otras fueron inducidas por los tratamientos o por la salinidad.

Palabras clave: enzimas antioxidantes, lino, peroxidación de lípidos, perfil de proteínas, salinidad, α-Toco.

INTRODUCTION

Flax plant (*Linum usitatissimum* L.) is an important fiber plant and has been grown in many different countries as fiber, seed and double purpose plant (fibers and seeds) in the same time. It is grown in Egypt as an important economic industrial and dual purpose crop (El Hariri *et al.,* 1998).

It is well established that, salinity is one of the major problems of agriculture in arid and semiarid regions. Salt stress is considered as one of the most important abiotic factors limiting plant growth and productivity (Sairam and Tyagi, 2004). Salinity produces oxidative stress in plant tissues (Rout and Shaw, 2001). This stimulates the generation of reactive oxygen species, such as a singlet oxygen, superoxide anion, hydrogen peroxide and hydroxyl radical (Gossett *et al.,* 1994). The levels of reactive oxygen species are regulated by their rates of generation that is related with target substances such as proteins, lipids and/or nucleic acids, their potential rate of degradation and their rate of scavenging / buffering by enzymatic antioxidants (Hodge, 2003). Several enzymes are involved in the detoxification of reactive oxygen species, which resulted under salinity stress. Superoxide dismutase is the first defence enzyme which converts superoxide to H_2O_2. This can be scavenged by catalase and different classes of peroxidises (Sairam *et al.,* 2005).

In most plants, there is an increase in accumulation of amino acids and amines (e.g., proline, B-alanine, Glycine Betaine) in their tissues in response to salt stress. These compounds accumulating are differ between species and ranges from only one to several different compounds. Meanwhile, salinity induces inhibitory effects on the biosynthesis of free amino acids and opposite effect was observed on the biosynthesis of proline. This show the most common interpretation of proline accumulation, which acts as a cytoplasmic osmotic solute and as source of energy and nitrogen, so proline might play a role in the alleviation of salt stress (Venkatesan and Chellappan, 1998). Salinity stresses cause important modifications in gene expression (Soussi *et al.,* 2001). Gene expression is manifested by the appearance of new proteins, which are not present before the stimulation process. Salinity promotes the synthesis of salt stress – specific proteins (Hashem, 2000), many of these proteins were suggested to protect cell against adverse effect of salt stress (Guerrier, 1998).

To cope with stress and to avoid oxidative damage, plants have evolved a serious of enzymatic and non enzymatic antioxidant systems. From the latter ascorbic acid and α–tocopherols. Ascorbic acid is one of the most extensively studied antioxidants that have been detected in the majority of plant cell types, organelles and apoplast (Borland *et al.,* 2006). A fundamental role of ascorbic defence system in plant is protecting metabolic processes against H_2O_2 and other toxic derivatives of oxygen. The essential is a reductant and reacting with and/or scavenging many types of free radicals. Ascorbic acid reacts non–enzymatically with superoxide, hydrogen peroxide and singlet oxygen. (Pourcel, *et al.,* 2007).

α-Tocopherol (Vitamin E) are lipophilic antioxidants synthesized by all plants. α-Tocopherol interact with the polyunsaturated acyl groups of lipids, stabilize membranes which scavenge and quench various reactive oxygen species and lipid soluble by products of oxidative stress (Cvetkovska *et al.,* 2005; Noctor, 2006).

The aim of this work is to study the influence of foliar application of ascorbic acid and/or α-tocopherol on counteracting the deleterious effect of salinity on flax plant grown in Egypt.

MATERIALS AND METHODS

Experimental procedures

The current study was carried out to elucidate the roles of ascorbic acid and α-tocopherol on the oxidative defense systems of the three different cultivars of flax (*Linum usitatissimum* L.) grown under saline conditions. Also, to elucidate their roles in nullifications of all salt injuries. Seeds of flax three cultivars (Sakha 3, Giza 8 (Egyptian origin) and Ariane (French origin)) obtained from Agricultural Research Centre Giza, Egypt. The two applied substances (ascorbic acid (Asc) and α-tocopherol (α-Toco)) were supplied from Sigma Chemical Company, St. Louis, MO, USA. The experiment was carried out in the green house of National Research Centre, Cairo, Egypt during two successive seasons 2011/2012 and 2012/2013. The salt type used in irrigation was mainly the chloride mixture suggested by Stroganov (1962). The salt components of salt mixture are shown in Table 1.

Table 1. The component of salt mixture used for chloride salinization expressed as % of total salt content.

$MgSO_4$	$CaSO_4$	NaCl	$MgCl_2$	$CaCO_3$
10	1	78	2	9

The component of specific anions and cations in chloride mixture expressed as percentage of total milliequivalents.

Na^+	Mg^{+2}	Ca^{+2}	SO^{-2}	Cl^-	CO_3^{-2}
38	6	6	5	40	5

Three cultivar seeds of flax Sakha 3, Giza 8 (Egyptian origin) and Ariane (French origin) were separately sown on 7/11/2011 and 9/11/2012 in pots containing equal amounts of homogenous clay and sand (2:1). The plants were sprayed twice (after 30 and 45 days from sowing) with aqueous solutions of ascorbic acid or α-tocopherol (400 mg/L). The seedling (ten seedlings in each pot) were irrigated with saline water as described by Stroganov (1962) (2000,4000 and 6000mg/L) .

Five uniform seedlings per pot were left for experimentation. Phosphorus, potassium and ammonium fertilizers were added to the soil at the recommended doses.

Plant samples were taken after 60 days (at vegetative stage) from sowing for measurements of total soluble carbohydrates, proline, free amino acids, nucleic acids (DNA and RNA), lipid peroxidation, oxidative enzymes activities (superoxide dismutase, polyphenol oxidase, peroxidise and catalase) and protein profile.

Chemical analysis

Total soluble carbohydrates were extracted by the method of Homme *et al.,* (1992) and estimated according to Yemm and Willis (1954). Free amino acids were extracted according to Vartainan *et al.,* (1992) and estimated according to (Yemm and Cocking, 1955). Proline was extracted as free amino acid and assayed according to Bates *et al.,* (1973). Nucleic acid (DNA and RNA) was extracted following the method of Schmidt and Thannhauser (1945) with some modifications as described by Morse and Carter (1949). RNA was estimated colorimetrically according to (Dische, 1953) while DNA was estimated according to Burton (1956). The levels of lipid peroxidation were measured by determining the levels of malondialdhyde (MDA). Malondialdhyde is a product of lipid peroxidation and was assayed by thiobarbituric acid reactive substrates (TBARS) contents using the method of Stewart and Bewley (1980).

The method used for extracting the enzyme is that of MuKherjee and Choudhuri (1983). Super oxide dismutase (SOD, EC 1.12.1.1) activity measured according to the method of Dhindsa *et al.,* (1981). Polyphenol oxidase (PPO, EC 1.10.3.1) activity assayed using the method of Kar and Mishra (1976). Peroxidase (POX, EC 1.11.1.7) activity assayed using to the method of Bergmeyer (1974). Catalase (CAT, EC 1.11.1.6) activity assayed according to the method of Chen *et al.,* (2000). The enzyme activities calculated by Kong *et al.,* (1999).

Protein extraction was done according to Reuveni *et al.,* (1992) with some modifications. Electrophoretic protein profile of flax stems were analyzed according to sodium dodocylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) technique (Sheri *et al.,* (2000) which relates polypeptide maps, molecular protein markers, percentage of band intensity, molecular weight and mobility rate of each polypeptide to standard markers using gel protein analyzer version 3 (MEDIA CYBERNE TICE, USA).

Statistical analysis

The data were statistically analyzed on Factorial design according to Snedecor and Cochran (1980). Means were compared by least significant difference (LSD) at 5 % levels of probability (Duncan 1955).

RESULTS

Changes in total soluble carbohydrates

Data in Table 2 illustrate that increasing salinity levels up to 6000 mg/L increased total soluble carbohydrates in the three tested cultivars (Sakha 3, Giza 8 and Ariane) of flax stem as compared with the untreated controls. An average increase of about 41 %, 60 % and 80 % in Sakha 3, Giza 8 and Ariane cultivars respectively were obtained. Ariane cultivar accumulated more soluble carbohydrates in comparison with either Sakha 3 or Giza 8 cultivars. Ascorbic acid or α-tocopherol (400 mg/L) stimulated the accumulation of total soluble carbohydrates in the stems of flax three cultivars as compared with the corresponding salinity levels. More pronounced increase in total soluble carbohydrates was obtained with plants treated with ascorbic acid (Table 2).

Table 2. Effect of ascorbic acid and α-tocopherol on total soluble carbohydrates (TSC mg/100g dry wt), free amino acids (mg/100g dry wt) and proline (µg/100g dry wt) in flax cultivars grown under salinity stress.

Cultivar		Sakha 3			Giza 8			Ariane		
Salinity (mg/l)	Materials (400mg/ lmg)	TSC (mg/100g dry wt)	Free amino acids (mg/100g dry wt)	Proline (µg/100g dry wt)	TSC (mg/100g dry wt)	Free amino acids (mg/100g dry wt)	Proline (µg/100g dry wt)	TSC (mg/100 g dry wt)	Free amino acids (mg/100 g dry wt)	Proline (µg/100g dry wt)
0	0	1255	138.1	21.7	1022	156.1	22.9	3688	165.9	40.4
	Asc acid	1525	161.5	27.9	1240	163.7	35.7	3840	200.9	48.7
	α-Toc	1570	156.4	24.8	1136	150.2	31.2	4154	197.2	44.8
2000	0	1694	153.4	28.1	1359	155	29.2	6457	218	51.2
	Asc acid	1760	179.6	30.3	1656	159.3	43.6	8290	283.6	70.8
	α-Toc	1785	166.33	28.2	1760	149.4	36.4	6938	273.4	68.7
4000	0	1720	166.02	32.1	1458	152.9	33.5	6325	315.4	73.6
	Asc acid	1923	188.27	36.8	1676	154.7	46.4	9535	509.3	133.9
	α-Toc	1844	171.65	35.8	1721	152.4	44.2	7083	311.2	97.4
6000	0	1771	149.24	40.7	1636	146.7	40.3	6597	207.9	65.4
	Asc acid	2247	158.9	48.3	1787	149.7	51.3	8586	474.8	109.1
	α-Toc	1943	152.82	46.4	1753	146.3	48.3	6860	289.7	83.3
LSD		38.060	6.043	3.631	43.040	6.641	1.780	87.910	7.420	4.189

Changes in free amino acids

Salinity stress caused significant gradual increases in free amino acids with increases in salinity levels from 0 to 2000 to 4000 mg/L then decrease in magnitude but still more than control plants at 6000 mg/L in both Sakha 3 and Ariane cultivars. In contrary, non significant decrease at 2000 and 4000 mg/L and significant decrease at 6000 mg/L salinity level in free amino acids content of Giza 8 cultivar (Table 2). Furthermore, Asc or α-Toco significantly enhanced the stimulatory role of salt stress on production of free amino acids in Sakha 3, Giza 8 as well as Ariane cultivars. The highest level of free amino acids was found in plants treated with ascorbic acid than α–tocopherol.

Changes in proline contents

In flax plant, proline accumulation was observed in all stressed plants of three tested cultivars compared with those of control plants (Table 2). Data clearly show that the increase in proline level was much higher in stressed plants treated with ascorbic acid or α-tocopherol with increasing salinity level. These increases in proline contents of the three tested cultivars increased significantly (Table 2). However, stressed plants of Ariane cultivar accumulated much more proline compared to that of Sakha 3 and Giza 8.

Changes in nucleic acid contents

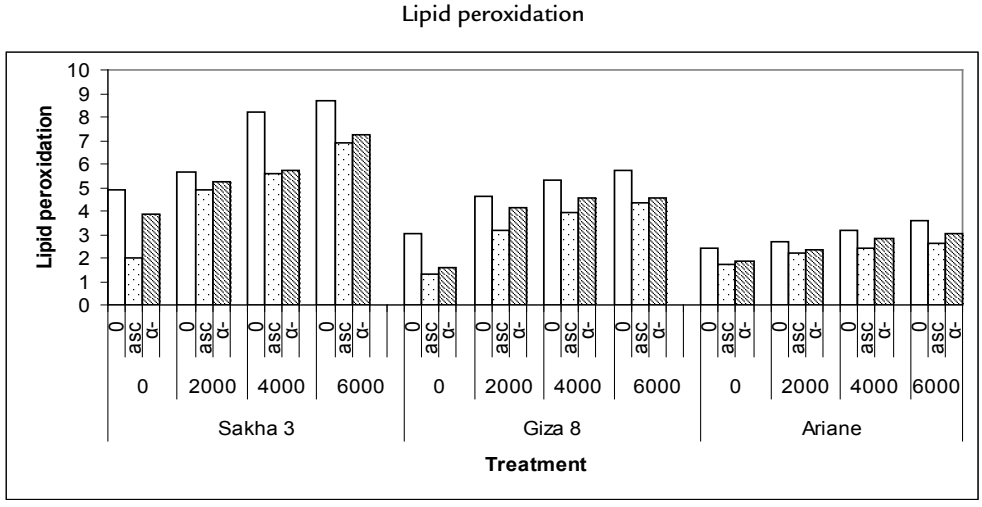
Data in Table 3 added also, that increasing salinity levels up to 6000 mg/L significantly decreased RNA and DNA contents in the three flax cultivars (Sakhs 3, Giza 8 and Ariane) as compared with the control plant. The rate of decrease in RNA and DNA were 52 % and 31 %, 44 % and 36 % and 37 % and 47 % in case of Sakha 3, Giza 8 and of Ariane, respectively. Exogenous application of ascorbic acid or α – tocopherol to three flax cultivars grown under different levels of salinity could over – come the reduction of RNA and DNA (Table 3).

Table 3. Effect of ascorbic acid and α-tocopherol on nucleic acid contents (DNA and RNA mg/100g dry wt) in flax cultivars grown under salinity stress.

Cultivar		Sakha 3		Giza 8		Ariane	
Salinity (mg/L)	Materials (400mg/lmg/L)	DNA mg/100 g dry wt.	RNA mg/100 g dry wt.	DNA mg/100 g dry wt.	RNA mg/100 g dry wt.	DNA mg/100 g dry wt.	RNA mg/100 g dry wt.
0	0	158.9	672.5	196.5	497.2	202.7	658.4
	Asc acid	252.5	790	246.9	653.9	375.7	794.4
	α-Toc	226.7	735.4	202.3	613.5	349.6	733.3
2000	0	145.6	605.4	177.1	455.9	170.7	617.1
	Asc acid	257.9	676.4	194.1	526	253.1	616.4
	α-Toc	213.6	641	187.5	523.4	261.1	611.4
4000	0	144	519.2	157.6	335.7	135.2	563.6
	Asc acid	175.7	582.3	175.5	433.9	206.9	617.3
	α-Toc	168.2	558.5	173.5	398.1	197.3	561.3
6000	0	109.3	323.9	140.8	278.6	108.3	416.9
	Asc acid	152.3	432.1	157.9	335.7	144.6	558.9
	α-Toc	144	427.4	147.3	333.5	142.4	521.4
LSD		7.343	4.189	3.010	5.450	4.079	6.529

Lipid peroxidation

Salinization significantly exerted an increase in lipid peroxidation reaching the maximum value at 6000 mg/L salinity level in the three tested flax cultivars. The effect was more pronounced in Sakha 3 than Giza 8 than Ariane. The inhibitory effect of foliar application of ascorbic acid or α-tocopherol on the accumulation of MDA was apparent in the three tested flax cultivars (Sakha 3, Giza 8 and Ariane).



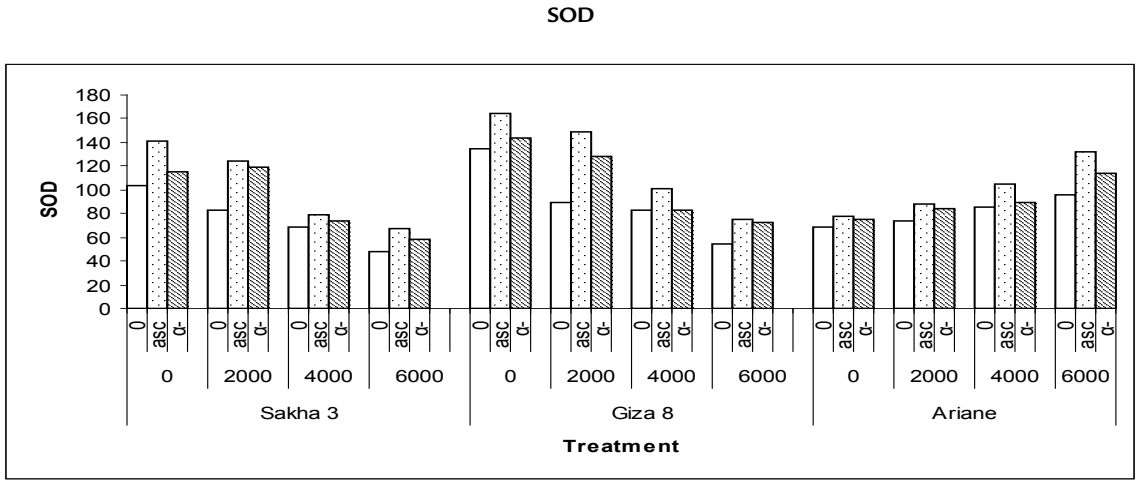
LSD at 5 %: 0.361, 0.241 and 0.053

Figure 1. Effect of ascorbic acid and α-tocopherol on lipid peroxidation (μmol/g fresh weight) of flax cultivars grown under salinity stress.

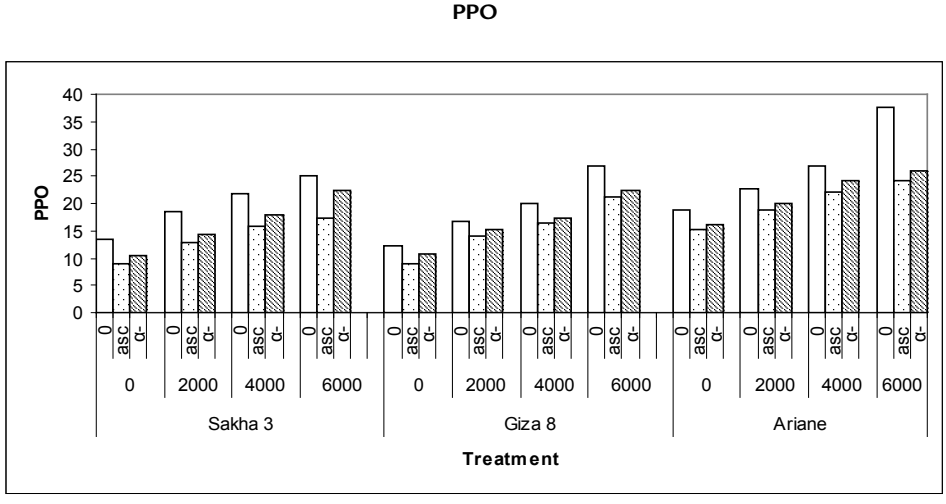
Enzyme activities

Polyphenol oxidase, peroxidise and catalase activities showed a gradual increases with the increase in salinity level in water of irrigation of the flax cultivars (Sakha 3, Giza 8 and Ariane) as shown in Fig (2). The effect of increasing salinity levels on superoxide dismutase activities of the three tested cultivars of flax plant as recorded in Fig (2), showed that Sakha 3 and Giza 8 cultivars (less salt tolerant) exhibited a decline in superoxide dismutase with the increase in salinity levels. On the other hand, salt tolerant cultivar Ariane showed an increase in the activity of superoxide dismutase with increasing salinity levels up to 6000 mg/L.

Foliar application of ascorbic acid or α-tocopherol in general, significantly decreased Polyphenol oxidase, peroxidise and catalase in Giza 8 and Ariane cultivars while catalase activity at Sakha 3 cultivar, which was increased as compared to the corresponding salinity levels. However, in the three tested cultivars superoxide dismutase activity increased by application of ascorbic acid and/or α-tocopherol as compared with the corresponding salinity levels.

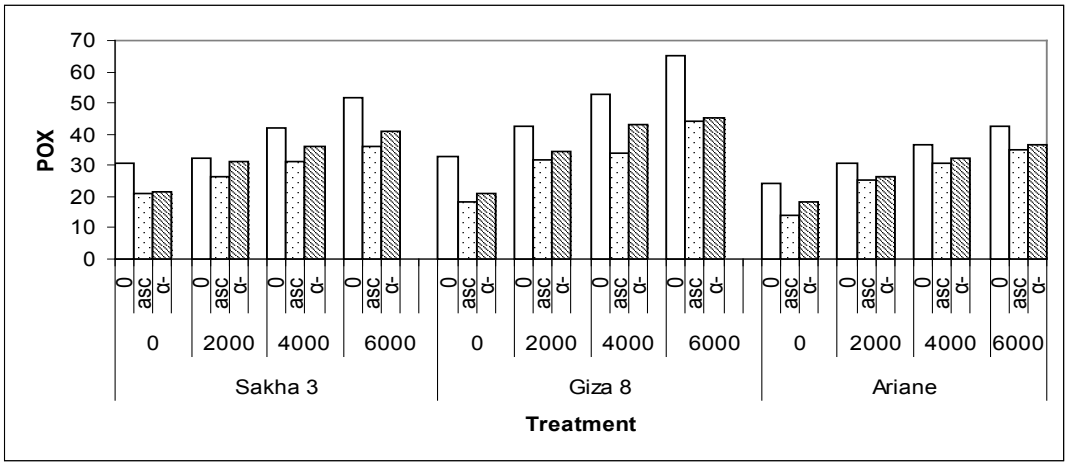


LSD at 5%: 6.48, 8.45 and 7.00



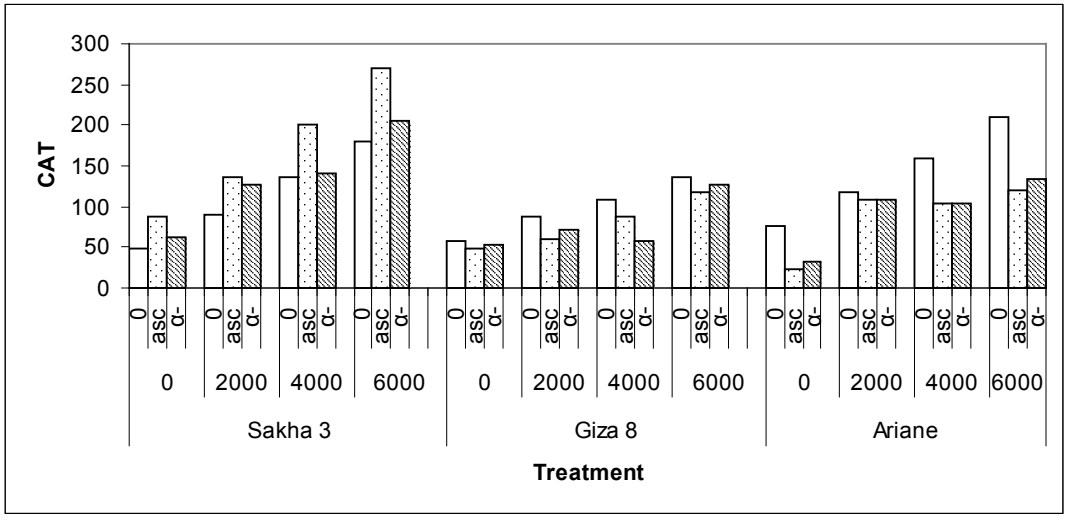
LSD at 5%: 0.365, 1.105 and 1.940

Figure 2. Effect of ascorbic acid and α-tocopherol on oxidative enzymes of flax (u activity/g fresh wt / hr) cultivars grown under salinity stress.



LSD at 5%: 1.701, 2.831 and 1.013

CAT



LSD at 5 %: 5.61, 6.12 and 1.94

Figure 2. Effect of ascorbic acid and α-tocopherol on oxidative enzymes of flax (u activity/g fresh wt / hr) cultivars grown under salinity stress. (continuatión)

Changes in protein electrophoretic patterns

The changes in protein electrophoretic patterns extracted from the leaves of three flax cultivars, Sakha 3, Giza 8 and Ariane grown under different salinity levels and treated with ascorbic acid and/or α–tocopherol are shown in (Fig. 1). Salt stress and vitamins treatments caused an induction of some polypeptides and other polypeptides disappeared. Flax plants grown only under salt caused changes in the levels of protein in the three cultivars with molecular weights of 127, 90, 76, 48, 40, 22, 20 and 17 KDa in Sakha 3, 144, 23, 17, 15 and 9 KDa in Giza 8 and 90, 60, 26, 23 and 13 KDa in Ariane. One protein with M wt. 90 was de novo synthesized in Sakha 3 and Ariane cultivars which grown under salinity stress. Also, it was observed the M wts. 76, 48 and 40 KDa were detected in Sakha 3 while disappeared from Giza 8 and Ariane. In addition, M wt 60 and 26 were detected in Ariane but disappeared from Sakha 3 and Giza 8.

Ascorbic acid and/or α – tocopherol treatment increase the intensity and density of salt responsive proteins which were detected in salinity treatments.

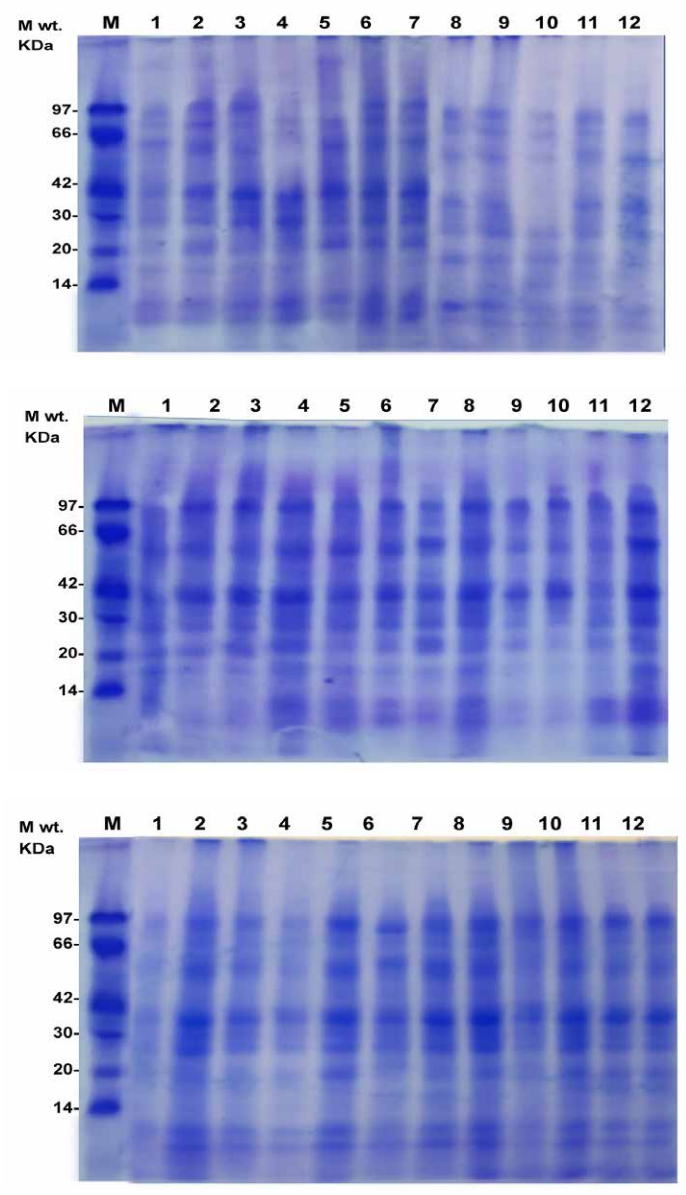


Plate 1. Electrograph of soluble protein pattern by one-dimensional SDS-PAGE showing the change of protein bands (marked by arrowheads) in response to ascorbic acid and α- tocopherol of flax shoots cultivars (A Sakha 3 , B Giza 8 and C Ariane) grown under salinity stress. Each lane contains equal amounts of protein extracted from shoots. Lane M Protein markers – Lane 1, control – Lane 2 ascorbic acid – Lane 3 α- tocopherol –Lane 4, 2000 mg/L salt–Lane 5, 2000 mg /L salt + ascorbic acid- Lane 6, 2000 mg/L salt + α-tocopherol Lane 7, 4000 mg/L salt–Lane 8, 4000 mg/L salt + ascorbic acid–Lane 9, 4000 mg /L salt +α tocopherol Lane10, 6000 mg/L salt -Lane 11,6000mg/L salt + ascorbic acid-Lane 12,6000 mg/L salt + α- tocopherol .

DISCUSSION

Data in Table 2 illustrate that increasing salinity levels up to 6000 mg/L, increased total soluble carbohydrates in the three tested cultivars (Sakha 3, Giza 8 and Ariane) of flax stem as compared with the untreated controls. Our obtained results are in agreement with those obtained by Khattab (2007), Hassanein *et al.,* (2009) and Rady *et al.,* (2011) on different plant species. The accumulation of organic solutes especially sugars are the main solutes involved in osmotic adjustment in glycophytic plants submitted to osmotic and saline stress (Amini and Ehasapour, 2005). Molecules like total soluble carbohydrates were discovered by empirical methods to protect biological macromolecules against the damaging effect of salinity (Sairam and Tyagi, 2004). Data obtained revealed that foliar treatment of the three tested cultivars with ascorbic acid or α-tocopherol (400 mg/l) stimulated the accumulation of total soluble carbohydrates as compared with the corresponding salinity levels. These results are in good agreement with that obtained by Chaparazadeh *et al.,* (2004), Hassanein *et al.,* (2009) and Hassan *et al.,* (2013). It could be concluded that, ascorbic acid and/or α–tocopherol foliar application generally stimulated the accumulation of total soluble carbohydrates in salt stressed flax plant either via increasing endogenous levels of certain phytohormones or by acting as activators of carbohydrates synthesis. Moreover, accumulation of carbohydrate play a key role in alleviating salinity stress, either via osmotic adjustment or by conferring some desiccation resistance to plant cells (Hassanein *et al.,* 2009). Also, these increments probably, may be attributed to the protective effects of ascorbic acid or α-tocopherol on photosynthetic systems. As well as ascorbic acid or α-tocopherol which play essential roles in plant metabolism and stress tolerance (Chaparazadeh *et al.,* 2004).

Amino acids are a putative osmoprotective solute leading to lowering osmotic potential in several tissues exposed to stress. Salinity stress caused significant gradual increases in free amino acids with increases in salinity levels. These results are in agreement with the results observed by wheat plant and Rady *et al.,* (2011) on sunflower plant, Sadak *et al.,* (2012) on sunflower plant and Sadak and Abd Elhamid (2013) on flax plant where, they concluded that salinity stress were capable of acting as activators of free amino acids accumulation. The accumulation of amino acids in flax plant exposed to stress may be attributed to the disturbance in amino acid metabolism. Furthermore, Asc or α-Toco significantly enhanced the stimulatory role of salt stress on production of free amino acids in Sakha 3, Giza 8 as well as Ariane cultivars. These results added support to the results obtained by Bassouny *et al.,* (2008) and Sadak *et al.,* (2010). Thus, it can be suggested that salt tolerance was manifested via activated proline synthesis and hydrolysis of protein into free amino acids, which act as osmoprotectants in the three tested cultivars of flax plant. It could be concluded that the inhibitory effect of salt stress on the flax cultivars was alleviated by foliar treatment of antioxidants through enhancing the biosynthesis of free amino acids and their incorporation into protein.

There is a positive correlation between increased cellular proline levels and the capacity of survival owing to the high environmental salinity under test. In flax plant, proline accumulation was observed in all stressed plants of three tested cultivars compared with those of control plants (Table 2). These results are in agreement with the results observed by, Alqurainy (2007), Bassouny *et al.,* (2008), Eid *et al.,* (2011), Rady *et al.,* (2011) and Sadak and Abd Elhamid (2013) on many plant species. These increases could be attributed to that proline may be reacted as an organic nitrogen reserve (Sairam and Tyagi, 2004). Osmolytes such as (proline) are known to play an important role in protecting macromolecules by stabilizing protein structure and/or scavenging ROS produced under stress conditions (Mitysik *et al.,* 2002). In addition, proline is a dominant organic molecule that acts as a mediator of osmotic adjustment under salinity stress, and/or stabilizer of sub-cellular structures which, could be considered as a sink for energy as well even a stress-related signal. Moreover, it is also involved in cell osmoregulation, protection of proteins during dehydration and can act as an enzymatic regular during stress conditions as mentioned by Rontein *et al.,* (2002).

Data clearly show that the increase in proline level was much higher in stressed plants treated with ascorbic acid or α-tocopherol with increasing salinity level. These increases in proline contents of the three tested cultivars increased significantly (Table 2). However, stressed plants of Ariane cultivar accumulated much more proline compared to that of Sakha 3 and Giza 8. These results may indicate that Ariane cultivar was more tolerant than other two test flax cultivars and this may be due to the varietal differences in flax tested cultivars. Similar results have been reached by Alqurainy (2007) and Bassouny *et al.,* (2008). In this respect, it could be concluded that, the inhibitory effect of salinity was alleviated by antioxidants applications to plant.

Data in Table 2 added also, that increasing salinity levels up to 6000 mg/l significantly decreased RNA and DNA contents in the three flax cultivars (Sakhs 3, Giza 8 and Ariane) as compared with the control plant. Similar results have been reported by Bekheta and El–Bassiouny (2005) on wheat as well Khattab (2007) on canola. It was postulated that, the contents of RNA and DNA in tomato decreased by NaCl salinized water due to its effect on the inhibition of synthesis and intensification of breakdown (Tsenov *et al.,* 1973). Salinization increases RNA ase activity in barley, tomato and pea (Tal, 1977). It was postulated that ROS which accumulated as a result of salt stress can damage essential membrane levels as well as nucleic acids. (Shalata and Neumann, 2001). Exogenous application of ascorbic acid or α – tocopherol to three flax cultivars grown under

different levels of salinity could over – come the reduction of RNA and DNA (Table 2) however, foliar treatment of used antioxidants promoted the synthesis of DNA and RNA and/ or prevented their degradation by nuclease enzymes. It was reported that ascorbic acid or α-tocopherol reacting directly or indirectly with reactive oxygen species, so contribute to maintain the integrity of cell structure such as proteins, lipids and nucleic acids from damage which induced by salt stress. (Cvetkovska *et al.,* 2005).

The extents of salt – induced oxidative damage, assessed by measuring the levels of malondialdehyd (MDA) were reported in Fig (1). Salinization significantly exerted an increase in lipid peroxidation in the three tested flax cultivars. There is a positive relation among the amount of lipid peroxidation and the degree of membrane damages which resulted from injurious salt stress. The effect was more pronounced in Sakha 3 than Giza 8 than Ariane. Ariane cultivar generally, had lower level of lipid peroxidation and this may be due to its genetical make up, however, it is of fiber flax varieties while both Sakha 3 and Giza 8 could be considered as double purpose plant (fibers and seeds). The low level of MDA in Ariane cultivar seems to enhance its tolerance. Similar results have been reported by Agarwal and Shaheen (2007), El-Beltagi *et al.,* (2008) and Farouk (2011). These increases may be attributed to salinity effect which could modify the membrane structure and stimulate O_2 production, which facilitates lipid peroxidation (Zhang and Kirkham, 1996). The inhibitory effect of foliar application of ascorbic acid or α-tocopherol on the accumulation of MDA was apparent in the three tested flax cultivars (Sakha 3, Giza 8 and Ariane). Alqurainy (2007) on bean and pea plants, Dolatabadian *et al.,* (2008) on canola plant and Dolatabadian and Saleh Jouneghani (2009) on bean plants, reported similar inhibitory effects of exogenous ascorbic acid. Also, Asada (1999) and Gupta and Datta (2004) found that α-tocopherol application suppressed membrane lipid peroxidation and plasma membrane permeability. The inhibitory effect of ascorbic acid or α-tocopherol on lipid peroxidation may be due to antioxidants effect which would inhibit stress – induced increases in the leakage of essential electrolytes following peroxidative damage to plasma membranes (Dolatabadian and Saleh Jouneghani, 2009).

Polyphenol oxidase (PPO), peroxidase (POX) and catalase (CAT) activities showed a gradual increases with the increase in salinity level in water of irrigation of the flax cultivars (Sakha 3, Giza 8 and Ariane) as shown in Fig (2). These increases in the activities of antioxidative enzymes under salt stress could be considered as an indicative of the increased production of ROS and a build – up of a protective mechanism to reduce oxidative damage triggered by stress experienced by plants as mentioned by Dolatabadian and Saleh Jouneghani (2009) and Rady *et al.,* (2011). Velikova *et al.,* (2000) where they reported that catalase and peroxidase are involved in overcoming the oxidative stress. Catalase activity was higher in salt tolerant Ariane than in the less tolerant Sakha 3 and Giza 8 respectively.

The effect of increasing salinity levels on superoxide dismutase (SOD) activities of the three tested cultivars of flax plant as recorded in Fig (2), showed that Sakha 3 and Giza 8 cultivars (less salt tolerant) exhibited a decline in SOD with the increase in salinity levels. On the other hand, salt tolerant cultivar Ariane showed an increase in the activity of SOD with increasing salinity levels up to 6000 mg/l. In this connection, Rout and Shaw (2001) and Hoque *et al.,* (2006) suggested that salt stress leads to a decrease in SOD activity in salt sensitive plants and this in turn showed an increase in salt tolerance one. Moreover, Dionisio – Sese and Tobita (1998) observed that the salt sensitive cultivars exhibited a decrease in superoxide dismutase (SOD) activity, while peroxidise activity was increased under high salinization. However, the salt tolerant rice cultivar showed only a slight increase and decrease in SOD and peroxidase activities respectively.

Multiple antioxidant enzyme systems are involved in the enzymatic scavenging of ROS. Superoxide dismutase (EC.1.15.1.1) reacts with the superoxide radicals to produce H_2O_2 which could be considered to be the first line of defense against ROS. Hydrogen peroxide is scavenged by catalase CAT (EC. 1.11.1.6) by breaking it directly to form water and oxygen and an increase in its activity is related to an increase in stress tolerance. Peroxidases decompose H_2O_2 by oxidation of phenolic compounds. The activities of peroxidase POX (EC. 1. 11. 1. 7) and polyphenol oxidase PPO (EC.1.10.3.1) were increased under salt stress. This was confirmed by our obtained results and the results of El–Bassiouny and Bekheta (2005); Khattab (2007); Agarwal and Shaheen (2007). Sairam and Srivastava (2002) reported that plant with high levels of enzymes either constitutive or inducible have been reported to have greater resistance to this oxidative damage.

Foliar application of ascorbic acid or α-tocopherol in general, significantly decreased PPO, POX and CAT in Giza 8 and Ariane cultivars while CAT activity at Sakha 3 cultivar, which was increased as compared to the corresponding salinity levels. However, in the three tested cultivars SOD activity increased by application of ascorbic acid and/ or α-tocopherol as compared with the corresponding salinity levels. Our obtained results are in good agreement with those obtained by El-Bassionny *et al.,* (2005) on faba bean and Dolatabadian and Saleh Jouneghani (2009) on common bean.

It could be concluded that, these reduction in enzyme activities could be attributed to antioxidants direct effects on scavenge ROS (O^{-2}), hydrogen peroxide (H_2O_2) and singlet oxygen (O_2) and /or preventing the enhancement of the mentioned activated oxygen species (Asada, 1999). In addition, Padh (1990) reported that ascorbic acid plays an

important role in preserving the activities of enzymes that contain prosthetic transition metal ions. Ascorbic acid acts as a primary substrate in cyclic pathway for enzyme detoxification of hydrogen peroxide (Shalata andNeumann, 2001).

The changes in protein electrophoretic patterns extracted from the leaves of three flax cultivars, Sakha 3, Giza 8 and Ariane grown under different salinity levels and treated with ascorbic acid and/or α–tocopherol are shown in (Plate 1). Salt stress and vitamins treatments caused an induction of some polypeptides and other polypeptides disappeared. Flax plants grown only under salt caused changes in the levels of protein in the three cultivars with molecular weights of 127, 90, 76, 48, 40, 22, 20 and 17 KDa in Sakha 3, 144, 23, 17, 15 and 9 KDa in Giza 8 and 90, 60, 26, 23 and 13 KDa in Ariane.

One protein band with M wt. 90 was de novo synthesized in Sakha 3 and Ariane cultivars which grown under salinity stress. In this respect, HSP 90 (a group of HSP) accumulates in response to drought and salt stress (Krishna *et al.,* 1995). Such protein is referred as stress associated proteins (SAP). Also, it was observed the protein bands with M wt. 76, 48 and 40 KDa were detected in Sakha 3 while disappeared from Giza 8 and Ariane. In addition, M wt 60 and 26 were detected in Ariane but disappeared from Sakha 3 and Giza 8. The protein band with M wt 40 KDa seems to be hydrin (Shukry, 2001). They have a protective role in survival under water stress due to their function as ion trap in dehydrating cells, sequestering ions as they become concentrated (Close and Lammers, 1993).

In addition, the protein with molecular weight 26 KDa seems to be osmotin as it expression under salinity stress was related to increased salt tolerance (Shukry, 2001; El–Bassiouny, 2005) in flax and wheat plants respectively. It has been suggested that, these proteins have an osmoprotection function (Dure, 1993) or protected cellular structures (Close and Lammers, 1993). It could be concluded that Ariane and Sakha 3 cultivars showed salt tolerance for the capacity of salt responsive proteins.In addition, the three cultivars had a band with molecular weight 23 KDa. The expression of these polypeptides was genetically regulated depending on the salt concentrations as well as the genetic differences in flax cultivars (El–Beltagi *et al.,* 2008). Also, a new protein bands with molecular weight 17 and 15 KDa were detected in Giza 8. In this respect, El–Bassiouny (2005) found that a 15 KDa protein was induced by salt treated in salt sensitive rice and wheat cultivars, respectively. In addition it is worth to note that Giza 8 had a band with a M wt 9 KDa at 4000 and 6000 mg/l salinity levels and also detected in Ariane in control and treated plants was appeared to be Ubquitin. It is a small heat stress protein consisting of 76 amino acid at 8.8 KDa (Kruse and Kloppstech, 1992). Ubiquitin appeared to protect protein from degradation by protease by tagging the protein (Hershko, 1988).

Ascorbic acid and/or α – tocopherol treatment increase the intensity and density of salt responsive proteins which were detected in salinity treatments.

CONCLUSION

In conclusion, the present results show that the salinity tolerant of Ariane cultivar as manifested by lower decrease in lipid peroxidation and increased the proline and free amino acid is associated with increased the capacity of salt responsive proteins than those of Sakha 3 and Giza 8. Exogenous application of ascorbic acid or α–tocopherol can mitigate the adverse effects of salinity through increasing the nucleic acid (DNA and RNA) is associated with higher antioxidant enzyme activities and salt responsive protein and reduce the lipid peroxidation.

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