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Role of Stigmasterol Treatment in Alleviating the Adverse Effects of Salt Stress in Flax Plant

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Abstract The results revealed that application of stigmasterol to salt - stressed flax plants causing significantly increased in growth parameters, membrane stability index, photosynthetic pigment and carbohydrate contents compared with salt-stressed plants untreated with stigmasterol. The level of antioxidant system components (catalase, ascorbic acid peroxidase) and reduced glutathione with increased in response to stigmasterol treatment. Enhanced antioxidant activities helped to decrease oxidative damage from salt and develop tolerance against salt stress in stigmasterol – treated flax plants. Stigmasterol treatment increase in the degree of salt tolerance by improvement of the membrane stability index, photosynthetic activity and carbohydrate contents. The data provided evidence that seeds soaking in stigmasterol reduced the adverse effects of salt stress on flax plants by stimulation of the antioxidant system as a stress protection mechanism.

Keywords: *Linum usitatissemum*; stigmasterol; salt stress; proline; antioxidant enzymes.
Abbreviations: APX-ascorbate peroxidase; CAT – catalase; chl a- chlorophyll a; chl b- chlorophyll b; GSH- reduced glutathione; GSSG- oxidized glutathione; LSD- least significant difference; MDA- malondialdehyde; MSI- membrane stability index; POD- peroxidase; SOD- superoxide dismutase.

Introduction

Flax (*Linum usitatissimum* L.) is a dicotyledonus plant from the family Linaceae. It is an important source of natural fibers and industrial oil, and has the potential of meeting edible oil and protein deficiency (Green and Marshall, 1984). It is rich in polyunsaturated fatty acids, particularly alpha-linolenic acid, the essential omega-3 fatty acid and linoleic acid, the essential omega-6 fatty acid. These two polyunsaturated fatty acids are essential for human, human bodies cannot manufacture them. These two fatty acids must be consumed in human diets (Morris, 2003). In Egypt, flax plants are cultivated for a dual purpose (seeds for oil and stem for fiber). The cultivated area through the last

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20 years was decreased from 60.000 to 30.000 feddan due to great competition of other economic winter crops resulting in a gap between production and consumption (Hussein, 2007 and Ibrahim, 2009). Salinity is a major abiotic stress that reduces the yield of a wide variety of crops (Ashraf and Foolad, 2007). Worldwide, 100 million ha or 5% of the arable land is adversely affected by high salt concentrations that reduce crop growth and yield (Ghassemi *et al*., 1995). Plants growing in saline environments exhibit various strategies at both the whole plant and cell level that allow them to overcome the salinity stress, The problems posed to higher plants by a saline environment results from osmotic stress due to the difficulty in absorbing water from soil of unusually high osmotic pressure, and ionic stress resulting from high concentrations of potentially toxic salt ions which lie above the limit to which most plants are adapted for optimum growth. Both of these components of salt stress affect a growing plant by causing changes in membrane chemistry, cell and plant water status, enzyme activities, protein synthesis and gene expression (Alamgir *et al*., 2008; Turkan and Demiral, 2009).Also under salt stress, shoot and root length, fresh and dry masses, water and pigment contents were decreased significantly by increasing salinity level (Azooz, 2009; Cha-um *et al*., 2009 and Azzedine *et al*., 2011). Cell membrane stability has been used as an efficient criterion to discriminate among crop cultivars with respect to degree of salt tolerance (Farooq and Azam, 2006, Jamil *et al*., 2008). In addition, Collado *et al*. (2010) reported that salinity stress decreased membrane stability index (MSI) of Zea mays genotypes. In similar trend, when chickpea plants were subjected to salt stress (100 mM NaCl), this stress was reported to enhance electrolyte leakage and lipid peroxidation in leaves (Sheokand *et al.,* 2008). Qing and Guo (1999) showed that, in sweet potato leaves, with increasing NaCl concentration, the number of chloroplasts decreased, and chlorophyll a and b synthesis were inhibited by high salinity treatments. Moreover, in tomato (Hajer *et al*., 2006), in pea (Hussein *et al.*, 2006) and in maize (Hassanein *et al*., 2009a) increasing salinity resulted in strong reduction in chlorophyll a, chlorophyll b and carotinoid contents. In addition, as a result of limited CO_2 fixation, reactive oxygen species, such as superoxide (O^{-}_{2}), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^{-}), can be overproduced in the chloroplasts and other organelles, thus leading to disruption of cellular metabolism through membrane lipid peroxidation, protein oxidation, enzyme inhibition and damage to nucleic acids (Prakash *et al*., 2011; Sabra *et al*., 2012). To detoxify the active oxygen species, a highly efficient antioxidant defense system is induced in plant cells.

Antioxidants can be divided into two classes (1) non enzymatic constituents, including lipid- soluble and membrane- associated tocopherols, water- soluble reductants, ascorbic acid and glutathione, and (2) enzymatic

constituents, including superoxide dismutase (SOD) , catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX) and glutathione reductase (GR) (Eyidogan and Öz, 2007). There are several reports of increasing activity of antioxidant enzymes in plants subjected to salt stress (Meneguzzo and navari – Izzo, 1999; Hernandez *et al*., 2000). Activated oxygen compounds may also cause damage to photosynthetic apparatus (Heidari and Golpayegani, 2012).

Oxidative molecules initiate damage in the chloroplast and cause a cascade of damage including chlorophyll destruction, lipid peroxidation and protein loss (Zhang and Kirkham, 1994). Under salinity stress, the accumulation of sugars and other compatible solutes (e.g., proline) allows plant to maintain the cellular turgor pressure necessary for cell expansion under stress conditions; such compounds also act as osmoprotectants (Ruiz-Carrasco *et al*., 2011). Proline is also considered to be the only osmolyte able to scavenge free radicals, thereby ensuring membrane stabilization and preventing protein denaturation during severe osmotic stress (Szabados and Savoure, 2010). The protective role of proline in plants under water deficit or salinity conditions has been reported in several species (Ben Ahmed *et al*., 2010; Kumar *et al.,* 2010).

Recently, it was demonstrated that proline supplements enhance salt tolerance in olive plants by improving photosynthetic activity and increasing the activity of enzymes involved in the antioxidant defense system (Ben Ahmed *et al*., 2010). Stigmasterol is a structural component of the lipid core of cell membranes and is the precursor of numerous secondary metabolites, including plant steroid hormones, or as carriers in acyl, sugar and protein transport (Genus, 1978). Sterols play an important role in plant growth and development including cell expansion, vascular differentiation, etiolation and reproductive development (Clouse and Sasse, 1998 and Abd El-Wahed *et al*., 2001). A number of studies have provided evidence that fluctuation in the stigmasterol:sitosterol ratio plays a role in response to biotic and abiotic stresses (Arnqvist *et al.,* 2008). Exogenous application of stigmasterol could be expected to enhance the salt tolerance of crops and eventually improves crop productivity under high salinity. Therefore, the present investigation was undertaken to study the effect of different concentrations of NaCl on growth, cell membrane stability, photosynthetic pigment and carbohydrate contents, antioxidant enzyme activities, and glutathione and proline contents of flax plants, and to examine the role of stigmasterol as promising plant development regulatory substance to increase the salt tolerance of flax plants.

Materials and methods

Plant materials

The pure strains of flax (*Linum usitatissium* L.cv.Sahka2) seeds were obtained from the Agriculture Research Center, Giza, Egypt. Stigmasterol was purchased from MP Biomedicals, LLC. France.

Growth condition

The pot experiments were carried out under natural condition .Ten plastic pots (40 cm in diameter and 25 cm in depth) were used per treatment. Each pot contained 20 kg of a mixture of clay and sand (2:1 w/w). Phosphorus and potassium were added before sowing at a rate of 6.0 and 3.0g pot^{-1} in the form of calcium superphosphate (15.5 $\%P_2O_5$) and potassium sulfate (48 $\%K_2O$), respectively. Seeds of each plant under investigation were surface sterilized with 0.1 % mercuric chloride for 5 min and washed thoroughly with several changes of sterile distilled water. The seeds were then soaked overnight (12 hours) in either distilled water or 200 ppm of freshly prepared stigmasterol solution. Fifteen seeds per treatment (control and stigmasterol treatment) were sown in each pot at 3 cm depths. After emergence, the seedlings were thinned to ten healthy seedlings per pot. Pots were maintained in a green house under natural conditions of light with an 8 hours photoperiod and average 25/10 °C ± 3 ^{0}C day / night temperature. Twenty days after sowing, seedlings of the control plants and stigmasterol-treated plants were subjected to the desired salinization levels (0, 100, 150 and 200 mM NaCl).The plants were irrigated with water to rise the soil water holding capacity in each pot to 70% until the end of the experimental period. Samples from each treatment were collected at the vegetative stage (40 days- old plants), to determine growth characteristics (shoot and root length, number of leaves per plant, area of leaves per plant, fresh and dry weights of shoots and roots), electrolyte leakage, membrane stability index, photosynthetic pigment and proline contents in fresh leaves, carbohydrate contents in oven-dried leaves, and antioxidant levels in fresh leaves.

Electrolyte leakage

The total inorganic ions leakages from the leaves were measured by the method described by Sullivan and Ross (1979). Twenty leaf disks of 2ml diameter were placed in a boiling tube containing 10 ml deionized water. The tubes were heated at 45^{o}C (EC_a) and 55^{o}C (EC_b) for 30 min each in water

bath and the electrical conductivity (EC) was measured with a conductivity meter (ME977-C,Max Electronics, India). Subsequently the contents were boiled at 100^{o}C for 10 min and the EC was again recorded (EC_c). Electrolyte leakage was calculated by using the formula:

$$\text{Electrolyte leakage (\%)} = \frac{EC_b - EC_a}{EC_c} \times 100$$

Membrane Stability Index (MSI)

MSI was estimated by placing 200 mg of leaves in 10 ml double distilled water in two sets. One set was heated at 40^{o}C for 30 min in a water bath and the electrical conductivity (C1) was measured. The second set was boiled at 100^{o}C in a boiling water bath for 10 min and the conductivity (C_2) was measured; both conductivities were measured using a conductivity meter (ME977-C,Max Electronic, India). The MSI was calculated using the formula described by Sairam (1994).

$$MSI = [1 - (C_1/C_2) \times 100]$$

Photosynthetic pigments

The contents of the photosynthetic pigments; chlorophyll a (chl a), chlorophyll b(chl b) and carotenoids in fresh leaves were determined using the spectrophotometeric method recommended by Metzner *et al*. (1965) and described by Hassanein *et al*. (2009a). The concentration of each pigment (as μg/ml) was calculated using the following equations.

Chl a = 10.3 E_{663}- 0.918 E_{644}
Chl b = 19.7 E_{644} – 3.87 E_{663}
Carotenoids = 4.2 $E_{452.5}$ – (0.0264 chl a + 0.4260 chl b)

Finally, the pigment contents were expressed as μg g^{-1} dry weight (DW) of leaves.

Estimation of carbohydrate content

Soluble sugar was extracted from air-dried leaf tissue with 80% ethanol. One gram of the dried tissues was homogenized with 80 % ethanol then put on a boiling water bath for 15 minutes. After cooling, the extract was filtered and the filtrate was oven dried at 60^{o}C then dissolved in a known volume of water

to be ready for soluble sugar determination (Homme *et al*., 1992). The soluble sugars were determined by the anthrone sulphuric acid method described by Whistler *et al*. (1962). Polysaccharide content was determined in the dry residue left after extraction of soluble sugars. A known weight of dried material was added to 10 ml 1.5 N sulphuric acids in sugar tube with air reflux and heated at 100°C in a water bath for 6 hours (Hodge and Hofreiter, 1962). The hydrolysate was made up to a known volume to be ready for polysaccharide determination by the method of anthrone sulphuric acid reagent . Total carbohydrates content was calculated as the sum of the amounts of soluble sugars and polysaccharides of the same sample. All data were calculated as mg 100 g^{-1} dry weight of leaves.

Estimation of proline content

Free proline was extracted and determined in fresh leaves according to the method described by Bates *et al*. (1973). One gram of fresh leaves was homogenized in 10 ml of 3 % aqueous sulfosalicyclic acid, and filtrated. Two ml of the filtrate were mixed with 2 ml glacial acetic acid and 2 ml of acid ninhydrin reagent and heated for one hour at 100°C. The reaction mixture was extracted with 4 ml toluene, mixed vigorously in a test tube for 15-20 seconds. The chromophore containing toluene was aspired from the aqueous phase and warmed to room temperature. The absorbance was read at 520 nm using toluene as a blank. Proline concentration was determined and calculated as mg 100 g^{-1} DW of leaves.

Estimation of antioxidant system

Preparation of samples for enzyme extraction followed the method described by Mukherjee and Choudhury (1983). Superoxide dismutase (SOD; EC 1.15.1.1) activity was measured according to the method of Dhindsa et al. (1981) by determining its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT). One unit of SOD was defined as the amount of enzyme that caused half the maximum inhibition of NBT reduction to blue formazan at 560 nm under the experimental conditions. Catalase (CAT; EC 1.11.1.6) activity was assayed in a reaction mixture (3 ml) composed of phosphate buffer (50 mM, pH 7.0), 30 % (w/v) H_2O_2 and 0.5 ml of enzyme extract (Aebi, 1983). Catalase activity was estimated by the decrease of absorbance at 240 nm using a spectronic 601 UV spectrophotometer as a consequence of H_2O_2 consumption and was expressed according to Havir and Mellate (1987) as μM H_2O_2 oxidized g^{-1} fresh weight (FW) min^{-1}. Peroxidase (POD; EC 1.11.1.7) activity was determined using guaiacol. The reaction

mixture (3ml) was composed of 10 mM KH_2PO_4/K_2HPO_4 (pH 7.0), 10 mM H_2O_2, 20 mM guaiacol and 0.5 ml crude extract (Malik and Singh, 1980). The increase in absorbance due to the dehydrogenation of guaiacol was monitored at 470 nm (Klapheck *et al*., 1990) using a spectronic 601 UV spectrophotometer. Enzyme activity was expressed as the change in the optical density g^{-1} FW min-1.Ascorbate peroxidase (APX; EC 1. 11. 1. 11) activity was assayed according to Asada (1992) by measuring the decrease in absorbance using a Spectronic 601 UV spectrophotometer at 290 nm for 1 min as the result of oxidation of ascorbic acid .The level of lipid peroxidation was measured in terms of malondialdehyde (MDA) content using the method of Hodges et al. (1999). The MDA content was calculated using its absorption coefficient of 155 n mol^{-1} cm^{-1} and expressed as n mol (MDA) g^{-1}FW. Reduced glutathione (GSH) was extracted and measured by the method adopted by Tanaka *et al*.(1985).

Statistical analysis

All data were analyzed statistically by one-way ANOVA using the Statistical Package for Social Science (SPSS) program. Values in the tables indicate mean values of four independent determinations. The least significant difference (L.S.D) was used to test the difference between treatments; $p \leq 0.05$ and $p \leq 0.01$ were considered statistically significant and highly significant, respectively.

Results and discussions

Growth parameters

The effect of salinity and stigmasterol on growth parameters of flax plants are shown in Table1. In this respect, most growth parameters as height of shoot, root length, number of leaves per plant, area of leaves per plant, fresh and dry weights of shoots and roots of *Linum usitatissimum* plants were significantly reduced with increasing the salinity level, compared with non-salt-stressed plants. The inhibitory effect of salt stress was much more pronounced at the highest level of salinity. Moreover, shoot system appeared to be more sensitive to salinity than root system. These results are in agreement with those obtained by Hajer *et al.* (2006) on tomato, Stoeva and Kaymakanova (2008) on bean plants. Salts may also reduce plant growth by reducing cellular water potential or by interfering with nutrients uptake (Abo El-Kheir *et al.*, 2000) or by inhibition of water absorption as salinity increases the amount of work necessary to counteract osmotic and ionic stresses for normal cellular

maintenance, as a consequence, there are less energy left for growth requirements (El-Saidi, 1997 and Mekki and Orabi, 2007). The reduction in leaf area of salt-stressed Linum usitatissimum plants could be due to that, salinity decreased the turgor which may reduce both cell production and cell expansion within the leaves to reduce their transpiration rate and to avoid metabolic and cellular damage caused by low water potential (Aldesuquy *et al*., 2000; Saffan, 2008 and El-Sawy, 2009). In the present work the detected reduction in growth parameters of flax plants in response to all applied concentrations of NaCl (100, 150 and 200 mM) could be due to the decline in photosynthetic pigments concomitantly with decrease in total carbohydrates in salt stress flax plants compared with reference control .The deleterious effects of salinity on plant growth are associated with (1) low osmotic potential of soil solution (water stress), (2) nutritional imbalance, (3) specific ion effect (salt stress) or (4) a combination of these factors (Ashraf, 1994; El-Sawy, 2009).

Application of stigmasterol in the present work improved growth of Linum ustitisumum plants compared with untreated plants by causing significant increases in the values of leaf area, shoot and root length, fresh weight and dry matter of salt stressed plants. This could be attributed to the detected promotive effect of stigmasterol treatment on soluble sugar levels which is necessary for the turgor and increasing the efficiency of water uptake and protecting the photosynthetic pigments. It could be concluded from these results that stigmasterol act as growth stimulants which may play a role in mitigating the adverse effects of NaCl on plant growth and development through enhancing certain metabolic activities (Abd El-Wahed *et al*., 2001).

Table 1. Effect of different concentrations of NaCl either alone or in combination with stigmasterol on growth parameters of *Linum usitatissimum* L. plants each value is the mean of five replicates

Treatment	NaCl mM	Shoot length (cm)	Root length (cm)	No. of leaves per plant	Area of leaves (Cm^2)	Shoot weight (g) Fresh	Shoot weight (g) Dry	Root weight (g) Fresh	Root weight (g) Dry
Reference controls	00	29.3	8.4	63.0	22.0	1.048	0.132	0.066	0.014
	100	25.7	6.8	56.8	14.3	0.830	0.114	0.040	0.012
	150	22.4	5.4	47.8	9.9	0.530	0.062	0.026	0.011
	200	15.8	4.6	35.8	6.3	0.236	0.030	0.017	0.010
Stigmasterol 200 ppm	00	31..2*	8.8	66.4	29.6**	1.076	0.134	0.072	0.016
	100	29.02**	8.1*	62.8*	20.9**	0.860	0.122	0.050**	0.013
	150	25.1**	7.4**	57.4**	14.0**	0.592**	0.074*	0.032	0.011
	200	21.16**	5.2	40.6	8.36*	0.286**	0.042*	0.026	0.010
L.S.D. at 5 %		1.731	1.312	4.993	1.514	0.035	0.011	0.015	0.0006
L.S.D. at 1 %		2.384	1.807	6.877	2.085	0.049	0.015	0.021	0.0008

* Significant difference

** Highly significant difference as compared with reference controls.

Electrolyte leakage and membrane stability index (MSI)

Electrolyte leakage and membrane stability index enables to assess the injury of cell membrane. A different pattern of response was observed when electrolyte leakage and the MSI were analyzed in leaves of flax plants treated with different salinity levels and stigmasterol (Table 2). It is clear from the obtained results that, increasing the salinity levels caused a marked increase in electrolyte leakage and decrease in membrane stability index of flax plants compared with those of the reference controls. Application of stigmasterol corrects the stress- mediated damage to the plasma membrane, as was evident from the highly significant increase in membrane stability and highly significant decrease in membrane leakage of treated flax plants compared with those of the reference controls. Similar results were obtained by Hamada (1986) who found that brassinolide also modifies membrane structure/stability under stress conditions. In the present study one of the possible mechanisms for the improved membrane stability in response to stigmasterol treatment was the detected decrease in lipid peroxidation (as indicated by MDA content) in plants grown from seeds soaked in stigmasterol compared with plants grown from untreated seeds..Lower lipid peroxidation and higher membrane stability (lower ion leaching) have also been reported in salt - tolerant genotypes of rice (Tijen and Ismail, 2005) and sugarcane (Gomathi and Rakkiyapan, 2011).

Table 2. Effect of different concentrations of NaCl either alone or in combination with stigmasterol on membrane stability index (%) and electrical leakage (%) in leaves of *Linum usitatissimum* L. plants at the vegetative stage(40 days-old plants). Each value is the mean of three replicates

Treatments	NaCl mM	Membrane stability	Electrical leakage
Reference controls	00	85.7	11.47
	100	70.3	13.54
	150	66.1	13.58
	200	61.6	14.73
Stigmasterol 200 ppm	00	86.4*	3.37**
	100	79.6**	6.15**
	150	71.9**	8.71**
	200	69.5**	10.86**
L.S.D. at 5 %		0.753	0.554
L.S.D. at 1 %		1.037	0.762

* Significant difference

** Highly significant difference as compared with reference controls

Photosynthetic pigments

The contents of photosynthetically active pigments (chl a, chl b and carotenoids) estimated in leaves of flax plants at the vegetative stage are shown in Table (3). The present results showed that, the contents of photosynthetic pigments (chlorophyll a, chlorophyll b, chlorophyll a/b ratio, carotenoids and total pigments) were significantly reduced with increasing salinity levels in flax plants compared with those of the non-salt- stressed plants These results are parallel with those of Jaleel *et al*. (2008a), who found that, at low salinity regimes, a slight decrease was noted in chlorophyll a, chlorophyll b and total chlorophyll contents, but under high salinity conditions a significant reduction in the content of these pigments was observed and the chl a: chl b ratio also differed significantly under salinity stress. The decrease in chlorophyll content in salt stressed flax plants concomitantly with the increase in proline level (Table 5) led to the suggestion that nitrogen might be shifted to the synthesis of proline instead of chlorophyll (Da La Rosa-Ibarra and Maiti, 1995). In addition, Djanaguiraman and Ramadass (2004) ascribed the suppressed pigment content in rice plant under salt stress to increased activity of chlorophyllase enzyme or disruption of the fine structure of the chloroplast as well as instability of pigment protein complex. In the present study application of stigmasterol can alleviate the damage effects of salt stress on photosynthetic pigment contents compared with those of the reference controls. These results in leaves of treated flax plants were in good aggrement with the increase in growth rate as well as to the increase in carbohydrate contents. In this respect, Kalinich *et al*. (1985) stated that spray application of stigmasterol enhanced the photosynthetic apparatus and enzyme activity in beans. In addition, Abd El-Wahed (2001) found that, the contents of the photosynthetic pigments (chl a, chl b and carotene) were increased in maize as sitosterol concentration increased.

Table 3. Effect of different concentrations of NaCl either alone or in combination with stigmasterol on photosynthetic pigment contents of *Linum usitatissimum* L. leaves. Values expressed as μg g^{-1} DW. Each value is the mean of three replicates

Treatment	NaCl mM	Ch. a	Chl. b	Ch.a+ Chl.b	Ch.a/Chl. b	Carotenoids	Total pigments
Reference controls	00	165.8	63.76	229.58	2.599	57.62	287.2
	100	97.43	52.67	150.1	1.849	47.41	197.51
	150	63.63	31.97	95.6	1.990	33.24	128.84
	200	54.16	27.96	82.12	1.937	32.06	114.18
Stigmasterol 200 ppm	00	182.49*	72.25**	254.74**	2.525	64.47*	319.21**
	100	175.25**	71.67**	246.92**	2.445**	51.76	298.68**
	150	148.70**	55.88**	204.58**	2.661**	46.44**	251.02**
	200	121.94**	45.02**	166.96**	2.708**	40.73**	207.69**

L.S.D. at 5 %	12.453	6.058	13.211	0.123	5.084	10.913
L.S.D. at 1 %	17.152	8.345	18.196	0.169	7.002	15.032

* Significant difference

** Highly significant difference as compared with reference controls.

Carbohydrate contents

The effects of salinity and stigmasterol on carbohydrate contents (soluble, insoluble and total carbohydrates) of flax plants are shown in Table(4) The contents of total soluble sugars, insoluble sugars and total carbohydrates decreased with increasing salinity level compared with those of non- salt-stressed plants. Such inhibition in the accumulation of carbohydrate was recorded by other authors (Kafiet *et al*., 2008; Hassanein *et al*., 2009 a). The decrease in carbohydrate contents, photosynthetic pigment contents and all the estimated growth parameters were directly proportional to the applied concentration of NaCl. Such results led to the conclusion that NaCl may inhibit photosynthetic activity and/or increase partial utilization of carbohydrates in other metabolic pathways .Moreover Hassanein *et al*. (2009a) attributed the overall reduction of growth parameters to higher sensitivity of photosystem II and Hill reaction activity to salinity stress which resulted in reduction of photosynthetic capacity in different saline stressed plants. Application of stigmasterol was found to partially alleviate the harmful effect of salt stress on different carbohydrate fractions by causing highly significant increases in soluble sugar, insoluble sugar and total carbohydrate contents in leaves of salt-treated flax plants compared with the reference control.

Table 4. Effect of different concentrations of NaCl either alone or in combination with stigmasterol on carbohydrate contents of *Linum usitatissimum* L. shoots at the vegetative stage. Values are expressed as mg glucose per 100g DW of leaves. Each value is the mean of three replicates

Treatment	NaCl mM	Total soluble sugar	Insoluble sugar	Total carbohydrate
Reference controls	00	1522.28	3693.70	5215.98
	100	996.56	3707.42	4703.98
	150	772.56	2985.13	3757.69
	200	269.71	2619.42	2889.13
Stigmasterol 200 ppm	00	1622.85**	7615.98**	9238.83**
	100	1051.42**	5897.13**	6948.55**
	150	895.99**	4767.99**	5663.98**
	200	539.42**	4237.70**	4777.12**
L.S.D. at 5 %		38.866	63.771	21.715
L.S.D. at 1 %		53.533	87.835	29.909

* Significant difference

** Highly significant difference as compared with reference controls.

In this connection, Abd El-Wahed (2000) and Abd El-Wahed (2001) found that, treatments of maize with stigmasterol, and sitosterol resulted in significant increases in total soluble and non-soluble sugars contents and accumulation of sucrose at the tasselling stage compared with the control. Abd El-Wahed and Gamal El-Din (2004) stated that, 100 mg/l stigmasterol strongly affected growth and consequently the biochemical constituents of leaves (total sugars, phenols and indoles), of which the contents were increased. In addition the enhancement by stigmasterol on carbohydrates biosynthesis, especially soluble sugars that are considered to be the principle organic osmotica in a number of glycophytes subjected to saline condition (Greenway and Munns, 1980) highlight another possible mechanism by which stigmasterol play a positive role in alleviation of the harmful effect of salt stress.

Antioxidant defense system

The results presented in Table(5) show the effect of different concentrations of NaCl either alone or in combination with stigmaserol on the activities of the antioxidant enzymes SOD, CAT, POD and APX, and the changes in GSH content and lipid peroxidation, as indicated by the accumulation of MDA, in flax plants at the vegetative stage. The activities of SOD, POD and APX showed progressively increased with increasing salinity level, whereas CAT activity significantly decreased with increasing NaCl concentration, compared with those of the non-salt-stressed plants. These results are in agreement with those of Hassanein *et al*. (2009 b) who observed that salt stress has increased the activities of leaf antioxidant enzymes in leaves of *Zea mays* plants. In addition Farag (2009) reported that, in pea (*Pisum sativum* cv. Puget), high concentrations of NaCl (110-130 mM) enhanced the activities of cytosolic Cu/Zn-SOD, and chloroplastic Cu/Zn-SOD. Increased activity of these antioxidant enzymes is considered to be a salt-tolerance mechanism in most plants (Hu *et al*., 2012). On the other hand the present results showed that salt stress caused a decrease in catalase activity, which might lead to accumulation of H_2O_2 to atoxic level. In this respect, Feierabend *et al*. (1992) showed that, under stress conditions inactivation of catalase is linked to H_2O_2 accumulation. Salt stress preferentially enhances H_2O_2 content and the activities of SOD and APX, but decreases CAT activity (Lee *et al*., 2001), a significant increase in endogenous H_2O_2 contents and a marked decline in catalase activity is reported during induced thermotolerance in mustard (Dat *et al.* 1998). Catalase deactivation by salt stress may be due to prevention of new enzyme synthesis (Feierabend and Dehne, 1996) or catalase photo inactivation (Polle, 1997). The GSH contents was highly significantly decreased in flax plants in responce to salinity compared with that of non-salt-

stressed plant .This results is in agreement with the finding of Hernandez *et al*. (2000).

The decreased GSH contents in flax leaves might be due to its oxidation to oxidized glutathione (GSSG). This conclusion is supported by the result of Nakano and Asada (1981) who reported that, GSH is oxidized to GSSG, under H_2O_2 treatment. The decrease in GSH content concomitant with decreased activity of CAT, in the present work supported the above conclusion.

Application of stigmasterol ameliorated the effect of salinity, reduced the activity of SOD and POD and increased the activity of CAT enzymes in *linum usitatissimum* plants. Anuradha and Rao (2007) reported that, Cd toxicity decreased the CAT activity in radish seedlings and addition of brassinosteroids increased its activity. CAT is an important oxidizing enzyme that helps in the removal of H_2O_2 and helps in detoxifying harmful metabolic products; its activity appears to be positively correlated with an increase in growth (Vardhini and Rao 2003). The reduced POD activity might be an indicator of removal of stressful conditions by brassinosteroids (Vardhini and Rao, 2003).

Malondialdehyde is a product of POD activity on unsaturated fatty acids in phospholipids, and lipid peroxidation is responsible for cell membrane damage (Halliwell and Gutteridge, 1985). The lipid peroxidation level as indicated by MDA accumulation increased significantly under salt stress, which suggested that oxidative damage due to salinization of *Linum usitatissimum* plants is not under the control of the antioxidative enzymes monitored in the present work. This result is in agreement with the finding of Dionisiases and Tobita (1998), who reported an increase in lipid peroxidation in rice leaves during salt stress. Sudhakar *et al*. (2001) reported that the level of lipid peroxidation, as indicated by MDA formation was high in a salt- sensitive cultivar of mulberry (*Morus alba*), where as a tolerant cultivar showed no change in MDA content under NaCl salinity. These indicate that *Linum ustitisumum* is salt- sensitive plants. In the present work showed that, the accumulation of MDA contents coupled with reduced plant growth under salt stress and this agreement with the studies of Li (2009) and Koca *et al.* (2007), they reported that growth reduction under salt stress in different cultivars is closely associated with increased in lipid peroxidation levels. Interestingly, MDA content in flax plants was significantly decreased in response to stigmasterol treatment, which reinforced the suggestion that stigmasterol treatment can ameliorate the stressful condition by increasing the stability of membranes in flax plants.

Table 5. Effect of different concentrations of NaCl either alone or in combination with stigmasterol on antioxidant enzymes activities and contents of reduced glutathione, malondialdehyde and proline in leaves of *Linum usitatissimum* L. plants each value is the mean of three replicates

Treatment	NaCl mM	SOD (unit mg^{-1} protein)	CAT $\times 10^2$ (μM H_2O_2 oxidized g^{-1} FW)	POD (change in optical density g^{-1} FW min^{-1})	APX (mM ascorbate oxidized g^{-1} FW min^{-1})	Reduced glutathione (μg g^{-1} FW)	Lipid-Peroxidation MDA (μM g^{-1} FW)	Proline (mg per 100 g DW)
Reference controls	00	2.36	9.26	1.90	0.186	0.65	1.56	23.57
	100	3.31	5.87	2.46	0.148	0.54	1.66	27.97
	150	3.58	4.25	3.16	0.223	0.52	1.93	33.52
	200	5.55	3.40	12.9	0.335	0.51	2.01	36.10
Stigmasterol 200 ppm	00	1.44*	12.8**	1.73	1.52**	0.72*	1.32**	15.34**
	100	1.95**	8.19*	2.16	0.290*	0.65**	1.46**	15.39**
	150	2.72*	6.47**	3.11	0.365*	0.60**	1.58**	18.05**
	200	5.35	5.36**	4.0**	0.496*	0.57*	1.94	19.13**
L.S.D. at 5 %		0.785	1.273	1.074	0.124	0.058	0.139	5.870
L.S.D. at 1 %		1.081	1.754	1.480	0.171	0.080	0.191	8.085

* Significant difference

** Highly significant difference as compared with reference controls.

Changes in proline content

Salt stress markedly increased the proline content in salt-treated plants compared with non-salt-stressed plants (Table 5). The magnitude of the increase was directly proportional to NaCI concentration. The accumulation of proline concomitant with increasing salinity in flax plants was in agreement with the results obtained by De-Lacerda *et al.* (2003) and Kavi *et al.* (2005) these reported that, proline accumulation in response to several types of environmental stress, such as exposure to salinity, protected the cell by balancing the osmotic strength of the cytosol with that of the vacuole and external environment. Proline accumulation could be a protective response, not only because of the osmoprotectant role of proline that prevents water deficit stress under high salinity, but also as a result of the radical scavenger and protein stabilization properties (Kuznetsov and Shevyakova, 1997; Ben Ahmed *et al*., 2010). In addition proline accumulation was reported to serve as nitrogen storage compound and protect cellular structure (Hare and Gress, 1997).

Stigmasterol treatment of plants grown under different concentrations of salt resulted in a significant reduction in proline content compared with those of the reference controls. It is also evident from the present study that the level of proline increased in flax plants treated with salinity and decreased with stigmasterol treatment. This finding might be explained by the fact that

stigmasterol alleviate the inhibitory effect of salinity and enhance the biosynthesis of other amino acids and their incorporation into protein.

Conclusion

Finally, it can be concluded that presoaking flax seeds in stigmasterol could ameliorate the adverse effects of salinity stress via the enhancement of enzymatic antioxidant system (CAT and APX) and non enzymatic system (GSH) and reducing oxidative damage (membrane integrity and MDA). Meanwhile, plant grown under salinity condition the defense mechanism were not enough to counteract the oxidative damage. The increase in the degree of salt tolerance induced by stigmasterol was also reflected in the improvement in the growth, photosynthetic pigments content and consequently the carbohydrate pool in the presence of salinity. Thus, our data provide evidence for the stimulatory effects of stigmasterol to induce salt tolerance in flax plants.

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