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Stigmasterol treatment increases salt stress tolerance of faba bean plants by enhancing antioxidant systems

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Abstract

The effect of different concentrations of NaCl (100, 150, and 200 mM) on cell membrane stability, photosynthetic pigment and carbohydrate contents, antioxidant enzymes activities, glutathione contents, and lipid peroxidation (malondialdehyde content) in faba bean (*Vicia faba*) leaves were investigated. In addition, the role of stigmasterol, a promising plant development regulatory substance in increasing tolerance to salt stress was evaluated. The results revealed that salt-stressed bean plants treated with stigmasterol had an increased membrane stability index, and 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) 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 faba bean plants. An increase in the degree of salt tolerance induced by stigmasterol was indicated by the improvement of the membrane stability index, photosynthetic activity and consequently the carbohydrate pool. The data provided evidence that stigmasterol treatment reduced the adverse effects of salt stress on faba bean plants, and might play a key role in providing stress tolerance by stimulation of the antioxidant system as a stress protection mechanism.

Keywords *Vicia faba*; stigmasterol; salt stress; soluble sugars; proline; antioxidant enzymes.
Abbreviations APX- ascorbate peroxidase; CAT - catalase; chl *a*- chlorophyll *a*; chl *b*- chlorophyll *b*; EC- electrical conductivity; GSH- reduced glutathione; GSSG- oxidized glutathione; LSD- least significant difference; MDA- malondialdehyde; MSI- membrane stability index; POD- peroxidase; SOD- superoxide dismutase.

Introduction

Faba bean (*Vicia faba*) is widely grown in the Mediterranean region as a source of protein for both human and animal nutrition (Crepon et al., 2010). The nutritional value of faba bean has been attributed to its high protein content, which ranges from 25% to 35%. The seeds are also a good source of sugars, minerals and vitamins; being particularly rich in calcium and iron, and the contents of thiamin, tocopherols, niacin and folic acid are high as compared with other grains (Larralde and Martinez, 1991). In addition, cultivation of faba bean leads to an increase in the concentration of soil nitrogenous compounds (Hungria and Vargas, 2000). In Egypt faba bean is cultivated over a large area of arable land; the approximate area of the cultivated land growing faba beans is 127, 195 ha (Sharan et al., 2002). According to FAO (Food and Agriculture Organization), production of dry faba bean in Egypt is estimated to be approximately 400,000 tons per annum. Although Egypt is the third highest producer of faba beans, the country imports a considerable quantity from China to satisfy the local requirement. 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 salinity stress. The problems posed to higher plants by a saline environment results from osmotic stress as a result of the difficulty of absorbing water from soil of unusually high osmotic pressure, and ionic stress resulting from concentrations of potentially toxic salt ions higher than 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). 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 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, peroxidase, ascorbate peroxidase (APX), and glutathione reductase (Eyidogan and Öz, 2007). Numerous studies report increased 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). In this respect, 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 was inhibited by high salinity treatment. In addition, in tomato (Hajer et al., 2006), pea (Hussein et al., 2006) and maize (Hassanein et al., 2009 a) increasing salinity resulted in a strong reduction in chlorophyll *a* and *b* and carotenoid contents. 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 Savouré, 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 development (Clouse and Sasse, 1998; Abd El-Wahed et al., 2001). Stigmasterol is considered to be one of the mostly free or conjugated sterols that play essential functions in plant growth (Genus, 1978). 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). Numerous attempts have been made to improve the salinity tolerance of a variety of crops by traditional breeding programs, but commercial success has been limited thus far. Exogenous stigmasterol application has been proposed not only as a convenient approach for unveiling its role in the salinity response, but it is also considered to be an effective approach to enhance the salt tolerance of crops and eventually improves crop productivity under high salinity. The objective of the current study was to investigate the effect of different concentrations of NaCl on cell membrane stability, photosynthetic pigment and carbohydrate contents, antioxidant enzyme activities, and glutathione and proline contents of faba bean plants, and to examine the utility of stigmasterol as a promising plant development regulatory substance to increase the salt tolerance of faba bean plants.

Results

Electrolyte leakage and membrane stability index

A different pattern of response was observed when electrolyte leakage and the MSI were analyzed in leaves of *V. faba* plants treated with different salinity levels and stigmasterol (Fig. 1). Application of different concentrations of NaCl caused a significant increase in electrolyte leakage compared with the control plants. Maximum electrolyte leakage was recorded in the plants exposed to 200 mM NaCl. However, treatment of the stressed plants with stigmasterol caused a significant decrease in the ionic leakage compared with those of the reference controls. In contrast, exposure of the plants to NaCl caused a decrease in the MSI. In contrast, treatment of faba bean plants with stigmasterol caused significant increases in the MSI by 6.15%, 12.77% and 11.90% in plants simultaneously treated with 100, 150 and 200 mM NaCl, respectively, compared to the control plants not treated with stigmasterol.

Changes in photosynthetic pigment contents

The contents of photosynthetically active pigments (chl *a*, chl *b* and carotenoids) estimated in leaves of *V. faba* plants at the vegetative stage are shown in Fig. 2. The contents of chl *a*, chl *b*, chl *a* + chl *b*, carotenoids and total pigments were gradually lowered with the rise in salinity level compared with non-salt-stressed plants. The highest inhibitory effect of salinity on chl *a*, chl *b*, chl *a* + chl *b*, carotenoid and total pigments was recorded at 200 mM NaCl. Soaking seeds in stigmasterol did not only alleviate the inhibitory effect of salinity on photosynthetic pigment contents, but also induced a significant stimulatory effect on the biosynthesis of pigment fractions compared with those of the corresponding salt-stressed plants. Moreover, the chl *a:* chl *b* ratio was significantly reduced in response to salt stress. Stigmasterol treatment significantly increased the chl *a*:chl *b* ratio compared with that of untreated plants subjected to the corresponding NaCl concentration. These results indicating that the protective effect of stigmasterol on chl *a* was stronger than its effect on chl *b*. The increase in content of total photosynthetic pigments in response to stigmasterol treatment ranged from 6% to 41.3% compared with the values of the reference control plants.

Changes in carbohydrate contents

The effects of salinity and stigmasterol on carbohydrate contents (soluble, insoluble and total carbohydrates) of *V. faba* plants are shown in Fig. 3. The contents of total soluble sugars, insoluble sugars and total carbohydrates decreased with increasing salinity level compared with those of non-salt-stressed plants. Soaking seeds in stigmasterol resulted, generally, in significant increases in the contents of soluble, insoluble and total carbohydrates in leaves of faba bean plants. The maximum total carbohydrate content was estimated to be increased by 150.6% in plants treated with 150 mM NaCl + stigmasterol compared with that of the reference control.

Antioxidant enzyme activities

The results presented in Table 1 show the effect of different concentrations of NaCl in control plants or in plants pre-treated with stigmasterol on the activities of the antioxidant enzymes SOD, CAT, POD and APX in *V. faba* 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. Stigmasterol treatment induced significant decreases in the activities of SOD and POD, and significant increases in the activities of CAT and APX, compared with those of the reference controls.

Reduced glutathione and malondialdehyde contents

The changes in GSH content and lipid peroxidation, as indicated by the accumulation of MDA, in *V. faba* plants subjected to different levels of salinity and stigmasterol are shown in Table 1. The GSH content gradually decreased with increasing NaCl concentration, whereas a gradual increase in lipid peroxidation was observed, compared with those of

Table 1. Effect of different concentrations of NaCl (0, 100, 150 and 200 mM) in control plants or in plants pre-treated with stigmasterol on antioxidant enzyme activities and contents of proline, reduced glutathione and malondialdehyde in leaves of *Vicia faba* plants. Each value is the mean of three replicates.

Treatment	NaCl (mM)	SOD (unit g^{-1} FW)	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	33.5 cd	4.86 a	3.11 cd	0.148 e	0.50 abc	1.35 e	9.762 c
	100	49.34 c	3.08 bc	3.29 c	0.223 de	0.43 bc	1.56 d	17.498 b
	150	101.28 a	2.61 c	5.60 a	0.260 cd	0.43 bc	2.24 b	20.524 b
	200	111.67 a	1.34 d	5.60 a	0.297 bcd	0.42 c	2.85 a	23.945 a
Stigmasterol (500 μM)	00	20.62 d	5.31 a	1.82 e	0.334 bc	0.58 a	1.33 e	9.183 c
	100	27.63 d	4.66 a	2.47 de	0.353 bc	0.50 abc	1.38 e	9.736 c
	150	70.64 b	3.71 b	4.80 b	0.390 b	0.52 ab	1.60 d	10.420 c
	200	83.36 b	1.86 d	4.92 b	0.558 a	0.52 ab	1.99 c	19.998 b
LSD ($p = 0.05$)		24.07	0.651	0.661	0.106	0.091	0.177	3.240

Values within a column with the same lower-case letters are not significantly different ($p = 0.05$).

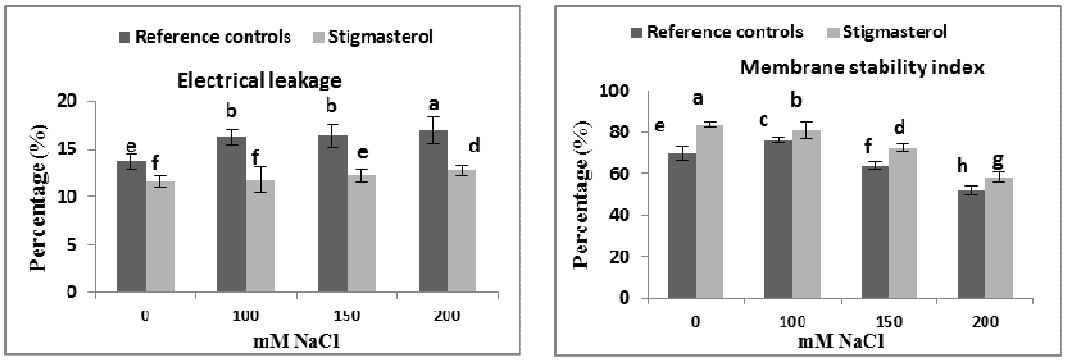

Fig 1. Effect of different concentrations of NaCl (0, 100, 150 and 200 mM) in control plants or in plants pre-treated with stigmasterol on membrane stability index (%) and electrical leakage (%) in leaves of *Vicia faba* plants at the vegetative stage (40 days-old plants). Each value is the mean of three replicates. Error bars represent the standard deviation. Treatments with the same lower-case letters were not significantly different based on a comparison of means with Duncan's multiple range test at $p = 0.05$

non-salt-stressed plants. The maximum reduction in GSH content (84% of that of the controls) and the maximum increase in MDA content (estimated to be 211.11% of that of the controls) were detected in plants treated with 200 mM NaCl. Stigmasterol treatment resulted in a non-significant increase in GSH content in salt-stressed plants except in plants irrigated with 200 mM NaCl, in which a significant increase of 23.81%, compared with that of the reference control, was detected. In contrast, stigmasterol treatment induced a highly significant decrease in MDA content, compared with that of the reference controls. The highest reduction in MDA content (estimated to be 30.18% compared with that of the reference control plants) was observed in plants treated with stigmasterol + 200 mM NaCl.

Changes in proline content

Salt stress markedly increased the proline content in salt-treated plants compared with non-salt-stressed plants (Table 1). The magnitude of the increase was directly proportional to NaCl concentration. 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. The minimum proline content was about 50.76%, compared with that of the reference controls, in plants treated with 150 mM NaCl + stigmasterol.

Discussion

Membrane Integrity

Membrane damage can be evaluated indirectly by measuring solute leakage (electrolyte leakage) from cells (Ekmekci et al., 2007) and the MSI (Ali et al., 2008). Increasing the salinity level in the present study caused a marked increase in electrolyte leakage and a decrease in the MSI of faba bean plants compared with those of the reference controls. Application of stigmasterol corrected the stress-mediated damage to the plasma membrane, as was evident from the significant increase in membrane stability and the significant decrease in membrane leakage of treated faba bean 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).

Photosynthetic pigments

The present results showed that the contents of photosynthetic pigments (chl *a*, chl *b*, chl *a*:chl *b* ratio, carotenoids and total pigments) were significantly reduced with increasing salinity level in faba bean plants compared with those of non-salt-stressed plants. These results are consistent with those of Jaleel et al. (2008), who found that, at low salinity regimes, a slight decrease was noted in chl *a*, chl *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 faba bean plants concomitant with the increase in proline content (Table 1) is consistent with the suggestion that nitrogen might be redirected 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 salt-stressed rice plants to increased activity of chlorophyllase or disruption of the fine structure of the chloroplast, as well as instability of the chloroplast membrane and pigment protein complex. In the present study application of stigmasterol alleviated the damage effects of salt stress on photosynthetic pigment contents by increasing the MSI (Fig. 1) compared with those of the reference controls. In agreement with these results, 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.

Carbohydrate contents

The carbohydrate fractions (soluble sugars, insoluble sugars and total carbohydrates) of leaves were markedly decreased in salt-stressed *V. faba* plants. Such inhibition in carbohydrate accumulation was recorded by other authors (Kafiet et al., 2008; Younis et al., 2008; Hassanein et al., 2009a,b). The decrease in carbohydrate and photosynthetic pigment contents were directly proportional to the applied concentration of NaCl. These results led to the conclusion that NaCl may inhibit photosynthetic activity or increase partial utilization of carbohydrates in other metabolic pathways. Application of stigmasterol generally stimulated the accumulation of carbohydrates in salt-treated *V. faba* plants and the inhibitory effects of salt stress were partially alleviated. In this connection, Abd El-Wahed (2000) and Abd El-Wahed (2001) found that treatment of maize with stigmasterol and sitosterol resulted in significant increases in total soluble and non-soluble sugar contents and accumulation of sucrose at the tasselling stage compared with the controls. 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 of carbohydrate biosynthesis, especially soluble sugars that are considered to be the principle organic osmotica in a number of glycophytes subjected to saline conditions (Greenway and Munns, 1980), highlight another possible mechanism by which stigmasterol plays a positive role in alleviation of the harmful effects of salt stress.

Antioxidant defense system

Salt stress in *V. faba* plants induced activation of antioxidant enzymes, such as SOD, POD and APX, in the leaves. These results are in agreement with those of Hassanein et al. (2009b), who observed that salt stress increased the activities of 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 (Ashraf, 2009; Hu et al., 2012). Previous studies showed that salt-tolerant cultivars generally have enhanced or higher constitutive antioxidant enzyme activity under salt stress compared with those of salt-sensitive cultivars. Such a trend has been demonstrated in numerous plant species, such as tomato (Mittova et al., 2004), *Crithmum maritimum* (Amor et al., 2006), *Azolla* (Masood et al., 2006), and *Medicago truncatula* (Mhadhbi et al., 2011). However, the present results showed that salt stress caused a decrease in CAT activity, which might lead to accumulation of H_2O_2 to a toxic level. In this regard, 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 content and a marked decline in CAT activity is reported during induced thermotolerance in mustard (Dat et al., 1998). Catalase deactivation by salt stress may be a result of prevention of new enzyme synthesis (Feierabend and Dehne, 1996). The GSH content was highly significantly decreased in faba bean plants in response to salinity compared with that of non-salt-stressed plants. This result is in agreement with the findings of Hernandez et al. (2000). The decreased GSH content in *V. faba* leaves might be because of its oxidation to oxidized glutathione (GSSG). This conclusion is supported by the results 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 supports the above conclusion. Application of stigmasterol ameliorated the effect of salinity, reduced the activity of SOD and POD, and increased the activity of CAT in *V. faba* plants. High activity of CAT in stigmasterol-treated plants under salt stress suggests that the treated plants possess a better scavenging ability. The decrease in POD activity in response to salt stress in the present study was consistent with the results reported by Mazorra et al. (2002). 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 as a result of salt stress in *V. faba* plants is not under the control of the antioxidative enzymes monitored in the present work. This result is in agreement with the findings of Dionisiases and Tobita (1998), who reported an increase in lipid peroxidation in rice leaves during salt stress. However, Sudhakar et al. (2001) reported that the level of lipid

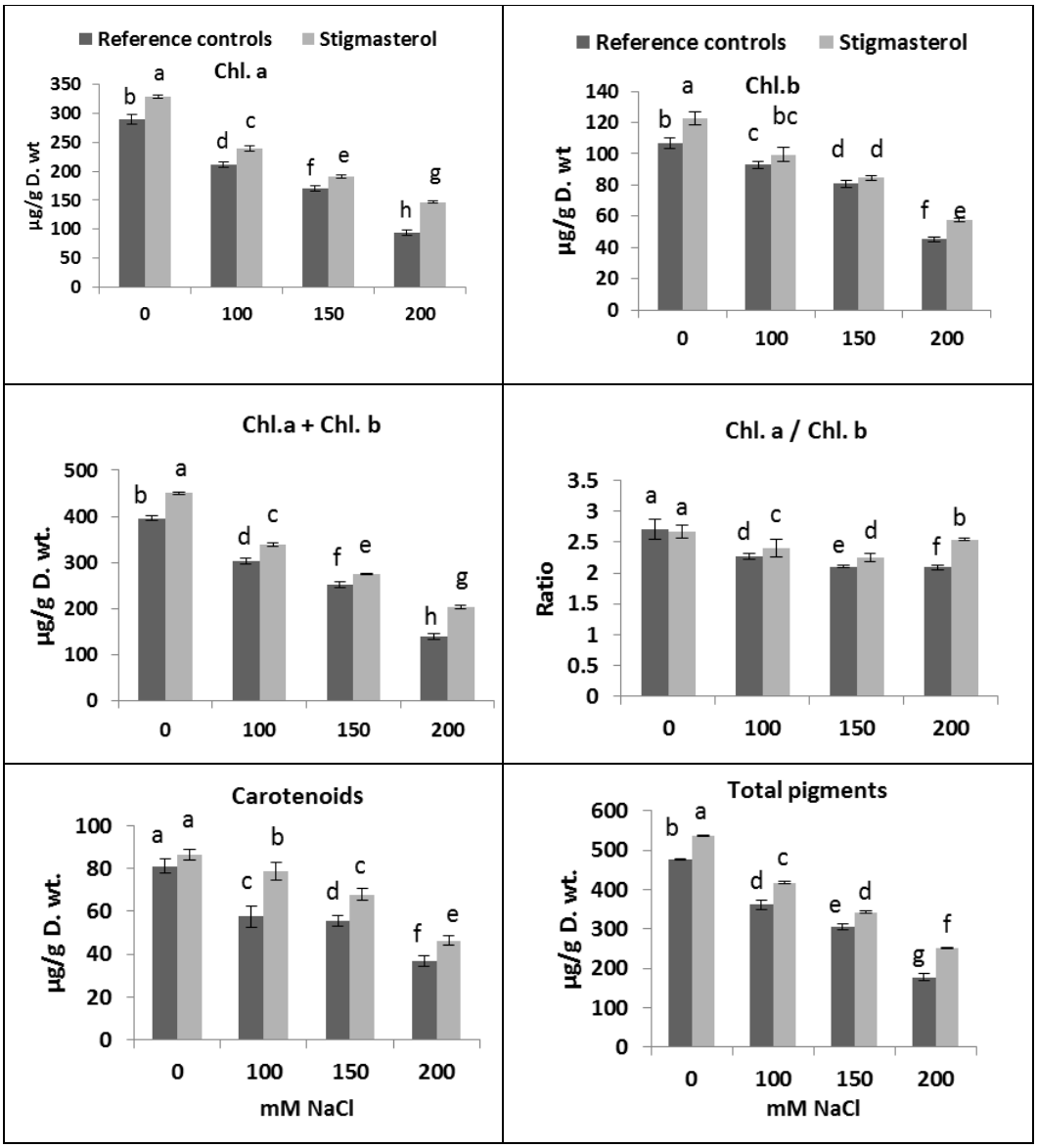

Fig 2. Effect of different concentrations of NaCl (0, 100, 150 and 200 mM) in control plants or in plants pre-treated with stigmasterol on photosynthetic pigment contents of *Vicia faba* leaves. Values are expressed as µg g^{-1} DW. Each value is the mean of three replicates. Error bars represent the standard deviation. Treatments with the same lower-case letters were not significantly different based on a comparison of means with Duncan's multiple range test at $p = 0.05$.

peroxidation, as indicated by MDA formation, was high in a salt-sensitive cultivar of mulberry (*Morus alba*), whereas a tolerant cultivar showed no change in MDA content under NaCl salinity. These findings indicate that *Vicia faba* is a salt-sensitive plant. Interestingly, MDA content in faba bean 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 *V. faba*.

Proline content

The accumulation of proline concomitant with increasing salinity in faba bean plants was in agreement with the results obtained by De-Lacerda et al. (2003) and Kavi et al. (2005). These authors 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 of proline (Kuznetsov and Shevyakova, 1997; Ben Ahmed et al., 2010). In addition, proline accumulation was reported to serve as a nitrogen storage compound and protect cellular structure (Hare and Gress, 1997). It is also evident from the present study that the level of proline increased in *V. faba* plants treated with salinity and decreased with stigmasterol treatment. This finding might be explained by the fact that stigmasterol

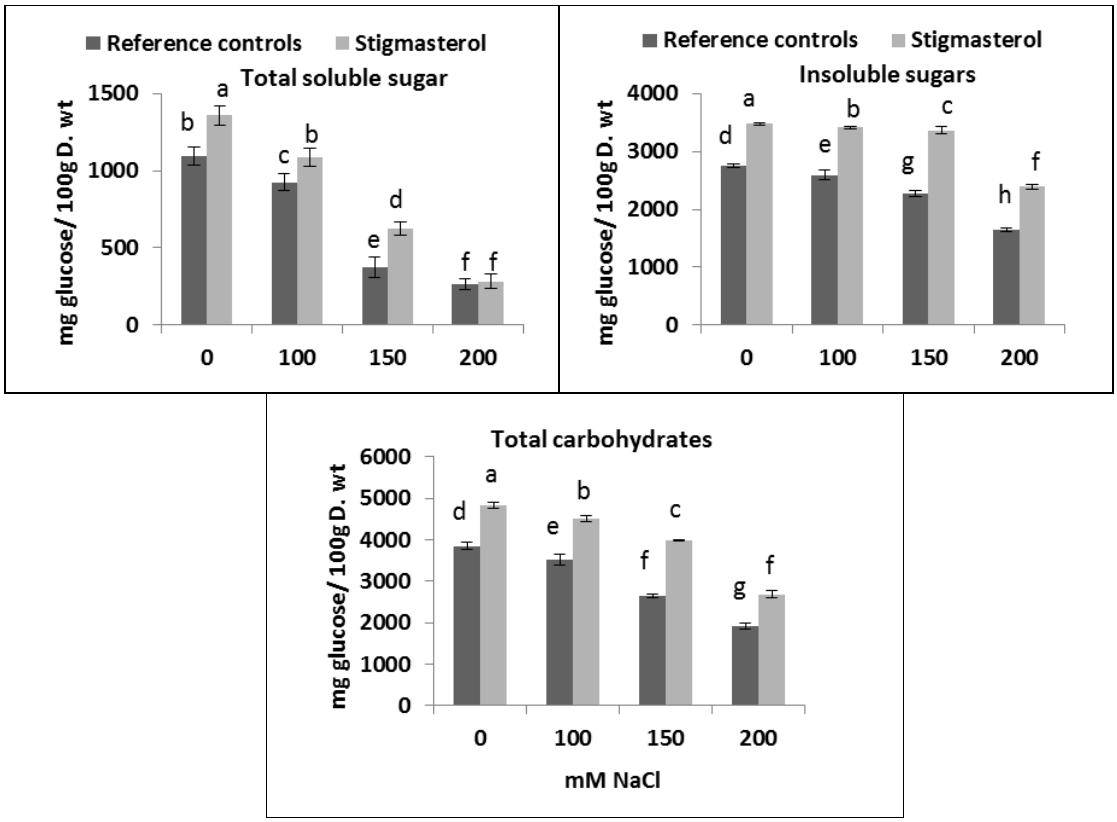

Fig 3. Effect of different concentrations of NaCl (0, 100, 150 and 200 mM) in control plants or in plants pre-treated with stigmasterol on carbohydrate contents of *Vicia faba* shoots at the vegetative stage. Values are expressed as mg glucose per 100 g DW of leaves. Each value is the mean of three replicates. Error bars represent the standard deviation. Treatments with the same lower-case letters were not significantly different based on a comparison of means with Duncan's multiple range test at $p = 0.05$.

enhances the biosynthesis of other amino acids and their incorporation into protein.

Materials and methods

Plant materials

Faba bean (*Vicia faba* L. cv. Sakha 1) seeds were obtained from the Agriculture Research Center, Giza, Egypt. Stigmasterol was purchased from MP Biomedicals LLC, Illkirch, France.

Growth conditions

A pot experiment was carried out under natural conditions. Ten plastic pots (40 cm in diameter, 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 3.0 g 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 h) in either distilled water or 500 µM freshly prepared stigmasterol solution (solution was prepared by dissolving stigmasterol in a minimum amount of chloroform then complete to the total volume by distilled water; the concentration was selected according to El Greedly and Mekki, 2005). Fifteen seeds per treatment (control and stigmasterol treatment) were sown in each pot at 3 cm depth. After emergence, the seedlings were thinned to five healthy seedlings per pot. Pots were maintained in a greenhouse under natural light conditions with an 8 h photoperiod and average 25/10 ± 3°C day/night temperatures. Twenty days after sowing, seedlings of the control plants and stigmasterol-treated plants were subjected to the desired salinization levels (0, 100, 150 or 200 mM NaCl). Thus, the different treatments were as follows: control (H_2O), 100, 150 and 200 mM NaCl, stigmasterol, 100 mM NaCl + stigmasterol, 150 mM NaCl + stigmasterol, and 200 mM NaCl + stigmasterol. The plants were irrigated to raise the soil waterholding 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 electrolyte leakage, the membrane stability index, phtotosynthetic pigment and proline contents in fresh leaves, carbohydrate contents in oven-dried leaves, and antioxidant levels in fresh leaves.

Methods

Electrolyte leakage

The total inorganic ion leakage from the leaves was measured by the method described by Sullivan and Ross (1979). Twenty leaf discs of 2 ml diameter were placed in a boiling tube containing 10 ml deionized water. The tubes were heated at 45°C (EC_a) and 55°C (EC_b) for 30 min each in a 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°C for 10 min and the EC was again recorded (EC_c). Electrolyte leakage was calculated with the formula:

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

Membrane stability index

The membrane stability index (MSI) was estimated by placing 200 mg of leaves in 10 ml double distilled water in two sets. One set was heated at 40°C for 30 min in a water bath and the electrical conductivity (C_1) was measured. The second set was boiled at 100°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 Electronics, India). The MSI was calculated using the formula described by Premchandra et al (1990) and modified 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 spectrophotometric 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 $= 4.2\ E_{452.5} - (0.0264 \text{ chl } a + 0.4260 \text{ chl } b)$

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 in a boiling water bath for 15 minutes. After cooling, the extract was filtered and the filtrate was oven dried at 60°C then dissolved in a known volume of water to be ready for soluble sugars determination (Homme et al, 1992). The soluble sugars were determined by the anthrone sulfuric acid method described by Scott and Melvin (1956). 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 1.5N sulphuric acid 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 in the same sample. All data were calculated as mg 100 g^{-1} DW of leaves.

Estimation of proline content

Free proline was extracted and determined in fresh leaves in accordance with the method of 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 $100g^{-1}$ DW of leaves.

Analysis of antioxidant system

Preparation of samples for enzyme extraction followed the method described by Mukherjee and Choudhurri (1983). Superoxide dismutase (SOD; EC 1.15.1.1) activity was measured in accordance with 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 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 in accordance with 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 (3 ml) 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 as a result of 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 in accordance with the method of 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 $nmol^{-1}$ cm^{-1} and expressed as nmol (MDA) g^{-1} FW. Reduced glutathione (GSH) was extracted and measured by the method adopted by Tanaka et al., (1985).

Statistical analysis

The experiment utilized a completely randomized design. Mean values were calculated from measurement of three replicates and standard deviations of the mean were calculated. All data were subjected to Duncan's multiple range test to discriminate significance (defined as $p = 0.05$). Duncan's multiple range test has high accuracy for mean comparison, and can comprise not only all treatments with the control group, but also all treatments together. All data were analyzed statistically by one-way ANOVA using the SPSS program (version 18.0).

Conclusion

The present study revealed that stigmasterol treatment (seeds pre-treated with 500 µM stigmasterol solution) induced augment of enzymatic antioxidant system (CAT and APX) and non-enzymatic antioxidant system (GSH), reducing

oxidative damage (membrane integrity and MDA) in NaCl stressful conditions. Meanwhile, in control plants submitted to NaCl stressful conditions the defense mechanisms 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 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 faba bean plants. The method of soaking seeds in stigmasterol is a simple and economic method for improvement of salt tolerance of plants and is environmentally safe.

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