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Effect Of Sitosterol On Growth, Metabolism And Protein Pattern Of Pepper (*Capsicum Annuum* L) Plants Grown Under Salt Stress Conditions

Sharifa S. Abu-Muriefah

Faculty of Science, Princess Nora Bint Abdulrahman University, Kingdom of Saudi Arabia

Corresponding author email: shiningday@hotmail.com

ABSTRACT: Experiments were carried out to investigate the effect of salinity stress on membrane stability, growth, chemical constituents, protein pattern and yield, and to examine whether salinity stress can be offset by the exogenous application of sitosterol on sweet pepper (Capsicum annuum L. cv. Orlando). Salinity stress (50, 100 or 200 mM) increased electrolyte leakage, decreased plant growth and marketable yield. Sitosterol (150 ppm) treatment as foliar spray treatment counteracted significantly the harmful effects of low and moderate salinity stress levels (50 and 100 mM) and partially counteracted the harmful effects under the highest salinity stress level (200 ppm). Low salinity stress increased chlorophyll content, soluble sugars and proline. The highest level of salt stress decreased the activities of super oxide dismutase (SOD), catalase (CA), peroxidase (POD), ascorbate peroxidase (APX) and glutathione reductase (GR) enzymes. While sitosterol enhanced membrane stability and improved the chemical constituents and the enzyme activity. The electrophoresis studies showed that some new protein bands were observed probably to increase plant tolerance against salt stress effect. These results provide support for the field application of sitosterol to alleviate the harmful effects of salinity on pepper plants.

Key words: Pepper, salinity stress, sitosterol, growth, enzyme activity.

INTRODUCTION

Pepper (Capsicum annuum L.) is an important agricultural crop, not only because of its economic importance, but also for the nutritional value of its fruits, it is an excellent source of natural colors and antioxidant compounds important for human health (Howard et al., 2000). Pepper is a moderately sensitive to salt stress (Lee, 2006), and it is grown under protected glasshouse conditions in temperate regions and in the open field under warm Mediterranean climates. Where it is grown in Saudi Arabian soils, it is frequently exposed to saline conditions brought about by saline irrigation water containing amounts of salts including sodium chloride (Kijne, 2003).

Salinity imposes stress conditions on crop plants (Hajer et al., 2006) and affect growth and chemical contents and has been shown to limit pepper yield (Paridam and Das, 2005). Salt stress severely inhibits plant growth for two reasons: first by an osmotic or water- deficit effect of salinity and second by a salt-specific or ion-excess effect of NaCl. Moreover, plants subject to salinity stress conditions produce cytotoxic activated oxygen that can seriously disrupt normal metabolism, through oxidative damage of lipids, proteins, and nucleic acids (Abbaspour, 2012). To defend against such oxidants, plants have evolved specific protective mechanisms, involving antioxidant molecules and enzymes that protect against the potentially-cytotoxic species of activated oxygen. Adaptation to salt stress requires alterations in gene expression and application of exogenous substances such as sitosterol.

Sitosterol is a phytosterol and 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 (Hassanein et al., 2012). Sterols play an important role in plant development including cell expansion, vascular differentiation, etiolation and reproductive development (Abd El-Wahed et al., 2001). Similar to the effect of brassinosteroids, sitosterol involved in the regulatory function of plant development, affected gene expression involved in cell expansion and cell division, vascular differentiation and other diverse developmental programs (Sasse, 2003). Sitosterol is known to influence permeability and fluidity characteristics of the plasma membrane and other organellar membranes in the plant (Senthil-Kumar et al., 2013). Interestingly, sitosterol also exhibit bacteriostatic or bactericidal activity against a broad range of gram-positive and gram-negative organisms, as well as Candida albicans (Hoffman, 2003). A number of studies have

provided evidence that fluctuation in the sitosterol ratio plays a role in response to biotic and abiotic stresses. (Arnqvist et al., 2008). In this respect, Griebel and Zeier (2010), working on Arabidopsis thaliana, found that, upon inoculation with pathogenic microbes, plants induce an array of metabolic changes that, potentially, contribute to induced resistance. When analyzing leaf lipid composition during the Arabidopsis thaliana - Pseudomonas syringae interaction, they found that accumulation of the phytosterol sitosterol is a significant plant metabolic process that occurs upon bacterial leaf infection. Sitosterol is synthesized from sitosterol by the cytochrome P450 CYP710A1 via C22 de-saturation. However, the role of sitosterol in plants during stress is still poorly understood.

The objective of this study was to investigate the effect of sitosterol on growth, chemical constituents, protein patterns and yield of sweet pepper (Capsicum annuum L.) plants grown under saline conditions in order to highlight the possible mechanisms by which sitosterol increases plant stress tolerance.

MATERIALS AND METHODS

Experiment preparation

Pot experiments were conducted under greenhouse conditions during summer seasons, to investigate the effect of salt stress on growth, yield and chemical constituents of sweet pepper (cv. Orlando) and to find out if sitosterol can counteract the harmful effect of salinity on plants.

Sweet pepper seeds were obtained from authorized agricultural company and were surface-sterilized with 0.1% mercuric chloride for 5 min and washed thoroughly with several changes of sterile distilled water. Five seeds per treatment (control and sitosterol treatment) were sown in each pot at 3 cm depth. After emergence, the seedlings were thinned to two 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, pepper seedlings were subjected to the desired salinization levels (0, 100, 150 or 200 mM NaCl). Salt was added in a gradual manner to prevent osmotic shock. Foliar application of sitosterol ST (150 ppm) was done twice, 30 and 37 days after sowing. Sitosterol solution was freshly prepared by dissolving sitosterol in a minimum amount of chloroform then complete to the total volume by distilled water; the concentration was selected according to Hashem et al. (2011). Eight treatments were conducted as follows: 1) control (H_2O), 2) 100 mM NaCl, 3) 150 mM NaCl and 4) 200 mM NaCl, 5) sitosterol, 6) 100 mM NaCl + sitosterol, 7) 150 mM NaCl +sitosterol, and 8) 200 mM NaCl + sitosterol. Plants were fertilized with Sangeral complete fertilizer (20%N : 20%P : 20% K plus essential micro nutrients) in two equal portions; the first one was added during the seedling stage and the second was added at the beginning of flowering stage. The plants were irrigated to raise the soil water holding capacity in each pot to 80% until the end of the experimental period.

Measurements

Sixty days after sowing, samples from each treatment were collected to determine growth characters, electrolyte leakage, membrane stability index, phtotosynthetic pigment, proline and antioxidant levels in fresh leaves contents in fresh leaves, soluble sugar contents in oven-dried leaves.

A) Growth characters

Plant height (cm); number of leaves/plant; leaf area (cm^2/plant) and shoot dry weight (g) were measured.

B) Determination of membrane characteristics

Lipid peroxidation (LP), electrolyte leakage (EL), and membrane stability index (MSI) were determined as follows:

Lipid Peroxidation

Lipid peroxidation was determined by measuring the amount of MDA according to Unyayar et al. (2006). About 0.5 g of leaf tissues from control and treated groups were cut into small pieces and homogenized by the addition of 5 ml of 5% trichloroacetic acid (TCA) solution. The homogenates were then transferred into fresh tubes and centrifuged at 12,000 rpm for 15 min at room temperature. Equal volumes of supernatant and 0.5% thiobarbituric acid (TBA) in 20% TCA solution were added into a new tube and boiled at 96 oC for 25 min. The tubes were transferred into ice-bath and then centrifuged at 10,000 rpm for 5 min. The absorbance of the supernatant was measured at 532 nm and corrected for non-specific turbidity by subtracting the absorbance at 600 nm, 0.5% TBA in 20% TCA solution was used as the blank. MDA contents were calculated using the

extinction coefficient of 155 M-1 cm-1. Values of MDA contents were taken from measurements of three independent samples, and SD of the means were calculated.

Electrolyte leakage

Ion leakage was determined as electrical conductivity (EC%) according to Hassanein et al. (2012). Leaf samples were cut into discs of uniform size and placed in 10 ml of double-distilled water at 40°C for 30 min, and its conductivity recorded (C1) using conductivity meter (Jenway 470 portable conductivity meter). Then it was kept in a boiling water bath (100°C) for 15 min and its conductivity also recorded (C2). The percentage of electrolyte leakage was calculated according to this formula: EC (%) = (C1/C2) × 100. Where C1 and C2 are the electrolyte conductivities measured before and after boiling, respectively.

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 (C1) was measured. The second set was boiled at 100°C in a boiling water bath for 10 min and the conductivity (C2) was measured; both conductivities were measured using a conductivity meter (ME977-C, Max Electronics, India). The MSI was calculated using the formula described by Sairam (1994):

$$MSI = [1 - (C1/C2)] \times 100$$

C) Determination of Photosynthetic pigments

The photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoids) were determined spectrophotometrically according to Metzner et al. (1965). A known fresh weight of leaves was homogenized in 85% aqueous acetone for 5 min. The homogenate was centrifuged and the supernatant was made up to known volume with 85% acetone and measured against a blank of pure 85% aqueous acetone at 3 wavelengths of 452.5, 644 and 663 nm. Taking into consideration the dilution made, it was possible to determine the concentrations of the pigment fractions (chlorophyll a, chlorophyll b and carotenoids) as g /ml using the following equations:

Chlorophyll a = 10.3 E 663 - 0.918 E 644

Chlorophyll b = 19.7 E 644 - 3.87 E 663

Carotenoids = 4.2 E 452.5 - (0.0264 chl a + 0.426 chl b).

Pigments then were calculated on the bases of mg/g fwt.

D) Determination of soluble sugars

Soluble sugar was extracted from 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 60oC then dissolved in a known volume of water to be ready for soluble sugars determination. The soluble sugars were determined by the anthrone sulfuric acid method described by Scott and Melvin (1953). Briefly, One ml of the extract was mixed with 9 ml of anthrone sulphuric acid reagent in a test tube and heated for 7 min at 100°C. The absorbance was read spectrophotometrically (Spectronic Genesys ZPC, Rochester, NY, USA) at 620 nm, against a blank containing only distilled water and anthrone reagent.All data were calculated as mg 100 g^{-1} DW of leaves.

E) Determination of free Proline

Free proline content was determined colorimetrically in aqueous sulfosalicylic acid as described by Bates et al. (1973). Briefly, lyophilized plant material (0.1 g) was homogenized in 10 ml of 3% aqueous sulfosalicylic acid and the homogenate filtered through Whatman #2 filter paper. Two ml filtrate was reacted with 2 ml acid-ninhydrin ($C_9H_6O_4$) and 2 ml of glacial acetic acid ($C_2H_4O_2$) in a test tube for 1 h at 100oC, and the reaction terminated in an ice bath. The reaction mixture was extracted with 4 ml toluene, mixed vigorously with a test tube stirrer for 15–20 s. After 1 h, toluene was added and absorbance at 520 nm was measured by using spectrophotometer (Shimadzu, RF-5301PC, Japan). The standard curve for proline was prepared by dissolving proline in 3% sulfosalicylic acid to cover the concentration range 0.5–10 μg ml-1. The proline concentration of the extract was determined from the standard curve and calculated on a dry weight basis.

F) Determination of antioxidant Enzyme activities

Enzyme extraction: The samples were prepared as described by Mukherjee and Choudhuri (1983). Fresh leaf samples were submersed for 5 min in liquid nitrogen.The frozen leaves were kept at -80°C for further analyses. Enzymes were extracted from 0.5 g leaf tissue using a mortar and pestle with 5 ml extraction buffer containing 50 mM potassium phosphate buffer pH 7.6 and 0.1 mM Na-EDTA. The homogenate was centrifuged at 15,000 g for 15 min and the supernatant fraction was used to assay for the various enzymes. All steps in the

preparation of enzyme extracts were performed at 4°C. Superoxide dismutase (SOD) was assayed according to Karanlık (2001), by monitoring the superoxide radical-induced nitro blue tetrazolium (NBT) reduction at 560 nm. One unit of SOD activity was defined as the amount of enzyme which causes 50% inhibition of the photochemical reduction of NBT. Catalase (CAT) activity was determined by monitoring the disappearance of H2O2 according to the method of Cakmak and Marschner (1992). Ascorbate peroxidase (APX) activity was determined by measuring the consumption of ascorbate by following absorbance at 290 nm. One unit of APX activity was defined as the amount of enzyme required to consume 1mol ascorbate min-1 (Cakmak and Marschner, 1992). Glutathione reductase (GR) activity was determined by measuring the enzyme-dependent oxidation of NADPH by following absorbance at 340 nm. One unit of GR activity was defined as the amount of enzyme that oxidized 1 mol NADPH min-1 (Cakmak and Marschner, 1992).

G) Protein patterns

Leaf samples were homogenized and extracted in 50 mM sodium phosphate buffer (pH 7.5). Protein samples were prepared by mixing the extract with 2X SDS-PAGE treatment buffer and boiled for 4 min. The denaturated protein samples were analyzed by vertical one dimensional Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE); Protein bands in the gel were visualized by a Coomassie Brilliant Blue R-250 (CB) with Bismarck Brown R (BBR) according to Choi et al. (1999).

H) Yield and its components

Fruit setting percentage was determined as the number of fruits set per number of flowers produced. Two fruit picking were taken from each treatment at 90 and 120 days from transplanting, total yield was calculated (summation of the two pickings).

For each treatment, number of fruits/plant; fresh weight of fruits/plant (g) and dry weight of fruits/ plant (g) were recorded.

Statistical analysis

The collected data were analyzed statistically using analysis of variance according to Snedecor and Cochran (1989) with the aid of COSTAT computer program. Treatment means were compared using the least significant differences (LSD) at 5% level.

RESULTS AND DISCUSSION

Vegetative growth

All growth characters of sweet pepper plants were decreased with increasing salinity stress levels with the greatest reduction observed at the highest salinity level (Table 1). It was clear that the most negative effect of salt stress was observed on the reduction of number of leaves and leaf area per plant. At salt concentration of 50 mM, the reductions of plant height, no. of leaves/plant and leaf area/plant were about 7%, 11% and 17%, respectively. While at 100 mM the reductions were nearly 16%, 21% and 36%, respectively. The corresponding reductions at 200 mM NaCl were about 25%, 37% and 56%, respectively. These reductions in growth attributes resulted in decreased shoot dry weight by about 27%, 39% and 56% at 50, 100 and 200 mM NaCl, respectively.

Data in the same table indicate that ST treatment improved the growth characters at all levels of salt stress and also control plants, it is therefore acting as growth stimulants. It seems that ST treatment could completely counteract the negative effect of low (50 mM) and medium (100 mM) salinity levels and could partially counteract the harmful effects of high (200 ppm) salt stress.

The inhibitory effects of salinity on growth of pepper plants reported in this study (Table 1) were typical of the effects of high soil salt availability and are probably due to decreased water absorption and disturbed metabolic processes leading to decreased meristematic activity and/or cell enlargement (Kaydan and Okut, 2007) coupled with an increase in

respiration rate resulting from higher energy requirements. Hussein et al. (2012) reported that there are two ways that salinity could retard growth, by damaging growth cells so that they cannot perform their functions or by limiting their supply of essential metabolites. Salinity stress is known to retard plant growth through its influence on several vital factors of plant metabolism, including osmotic adjustment (Sakr et al., 2013) nutrient uptake, protein and nucleic acid synthesis, photosynthesis, organic solute accumulation, enzyme activity, hormonal balance and reduced water availability at the cell level all of which result in reduced plant growth and ultimately reduced yield. Furthermore, increased salt content in the irrigation water may cause direct and indirect effects on leaf water relations and stomatal closure which influence CO2 ex-change and photosynthetic rate. Increased salt content in irrigation water may be directly toxic to plants, which in turn, lowered the accumulation of dry biomass in the plants (Hussein et al., 2012).

Simaei et al. (2011) attributed the depressing effects of salt stress on plant growth to an increase in reactive oxygen species which play an important role in damaging all classes of biologically important macromolecules including DNA and the generation of H_2O_2 and lipid hydro-peroxides which cause membrane changes. Reductions in fruit yield are largely attributable to decreases in the viability of pollen or the receptivity of the stigmatic surface (Sakr et al., 2004) and substantially increased abscission of flowers or young fruit due to ethylene induction by salinity. Other factors affecting cell division and cell expansion, such as tissue water status and the concentration of certain plant hormones e.g. ABA are also involved in the regulation fruit set under stress.

It is clear from the data in Table (1) that application of sitosterol improved the growth of pepper plants by causing significant increases in the values of shoot length, number of leaves per plant, area of leaves per plant and dry weight of shoots as compared with the corresponding controls. Similar results were obtained by Abd El-Wahed et al. (2001), working on wheat, who found that both sitosterol and spermidine caused stimulation of vegetative growth characteristics (plant height, leaf area, plant fresh and dry weight) and net assimilation rate and vascular bundles differentiation of wheat. This increase in growth parameters is, probably, caused by increasing the efficiency of water uptake and utilization, enhancing cell division and/or cell enlargement, resulting in longer shoots and increasing leaf area which, consequently, increased the dry matter of shoots, presumably, as a result of larger surface area available for anabolic activities (Hashem et al., 2011).

Lipid peroxidation and Electrolyte Leakage

A different pattern of response was observed when electrolyte leakage (EL), lipid peroxidation (LP) and the membrane stability index (MSI) were analyzed in leaves of Capsicum annuum plants treated with different levels of salinity and sitosterol (Table 2). Application of different concentrations of NaCl caused a significant increase in EL and LP, represented by the amount of Malon dialdehyde (MDA), compared with the control plants. The results illustrated remarkable increase in MDA content and ion leakage level in response to 100 and 200 mM NaCl. Maximum values of electrolyte leakage and lipid peroxidation were recorded in the plants exposed to 200 mM NaCl. However, treatment of the stressed plants with stigmasterl caused a significant decrease in the EL and LP compared with those of the reference controls. In contrast, exposure of the plants to NaCl caused a decrease in the MSI. While stigmasterl caused significant increases in the MSI of treatmed pepper plants.

Membrane damage can be evaluated indirectly by measuring solute leakage (electrolyte leakage) from cells and the MSI. The stimulation effect of saline water on the value of MDA and EL % might be attributed to injury of plasma membrane. That damage caused by ROS which could induce Lipid peroxidation and consequently Electrolyte leakage (Kassab et al., 2012). Reduction of MDA levels and EL% in response to stigmasterl treatments might be due to induction of antioxidant responses that protect the plants from oxidative damage, increased membrane stability and tolerance of plants which in turn enhanced scavenging of harmful free radicals (Sharhrtash et al., 2011) and elevated ca uptake that protects the plant from the oxidative damage. On the other side, application of stigmasterl seemed to correct the stress-mediated damage to the plasma membrane, as was evident from the significant increase in MSI and the significant decrease in EL of treated pepper plants compared with those of the reference controls. Similar results were obtained by Hassanein et al. (2012), who found that stigmasterl modifies membrane structure/ stability under stress conditions. In the present study one of the possible mechanisms for the improved membrane stability in response to stigmasterl treatments was the detected decrease in lipid peroxidation (as indicated by MDA content) in plants grown from seeds soaked in sitosterol 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 sugarcane (Gomathi and Rakkiyapan, 2011) and faba bean (Hassanein et al., 2012). In this concern, Senthil-Kumar et al. (2013) reported that sitosterol plays an important role in plasma membrane permeability, thus influencing leakage of cellular nutrients and ions into apoplast. In their study, they also investigated the role of sitosterol in imparting various abiotic stress tolerances in Arabidopsis.

Photosynthetic pigments

The obtained results showed that the contents of photosynthetic pigments such as chl a, chl b and carotenoids (Table 3), total chlorophyll (Fig.1) and chl a:chl b ratio were significantly reduced in pepper plants with increasing salinity level compared with those of non-salt-stressed plants in the absence of sitosterol treatments. Data in the table indicate clearly that the 50 mM salt treatment caused a slight increase in chl a, and chl b and carotenoid content as compared with salt untreated control. This observation was true either with or without sitosterol treatment. The most observed increase in total chlorophyll (Chl a + b) content was recorded at 50 mM NaCl + sitosterol (Fig. 1). In this regard, Zahra et al. (2010) found that chlorophyll content increased in tomato under low levels of salinity. One reason of that was the thicker leaves produced under salt stress. Increases in leaf thickness tended to compensate slightly for the negative effects of salinity on leaf

chlorophyll (Lui et al., 2007). The increased total chlorophyll under low levels of salinity recorded in this study is in agreement with the finding of Hussein et al. (2012) on pepper plants and Liu et al. (2007) on Aeluropus littoralis plants who found that salt stress increased Chl a and Chl b contents, but the Chl a/Chl b ratio declined, which implies the stimulation of Chl a accepted from NaCl was smaller than that of Chl b. This increase may be attributed to the thickness of the leaves under salt stress rather than to stimulation of pigment formation.

Increasing salinity levels from 50 to 100 and 200 mM significantly decreased chlorophyll a, chlorophyll b and carotene contents. At 200 mM of NaCl, Chla, Chl b and carotene decreased by about 46%, 55% and 51%, respectively, as compared with control treatment. Sitosterol treatments alleviated the deleterious effect of salinity on the chlorophyll content and increased chlorophyll a, b and carotene as compared to the control (Table 3). At no salt treatments, sitosterol caused an increase in Chl a by about 34%, Chl b by about 20% and carotene by about 26% of control. It seems that, at any salt treatment sitosterol decreases the harmful effect of salinity stress on chlorophyll content.

The decrease in chlorophyll content under salinity conditions was reported by Nazarbeygi et al. (2011) and might have been due to salt-induced increase in the activity of the chlorophyll degrading enzyme, chlorophylase (Yasar et al., 2008). Earlier studies reported that the reduction in leaf chlorophyll content of the plants grown in NaCl stress has been attributed to the destruction of chlorophyll pigments and instability of the pigment protein complex. Furthermore, increased salt content also interferes with protein synthesis and influences the structural component of chlorophyll. In this concern, a decrease in cholorophyll content (chl. a, b and total chl) of fennel (Rahimi et al., 2012) and faba bean (Azooz et al., 2013) under salt stress was observed. The decrease in chlorophyll content in salt-stressed pepper plants concomitant with the increase in proline content (discussed latter) is consistent with the suggestion that nitrogen might be redirected to the synthesis of proline instead of chlorophyll. In addition, Sevengor et al. (2011) 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 sitosterol alleviated the damage effects of salt stress on photosynthetic pigment contents by increasing the membrane stability index MSI (Table 1) compared with those of the reference controls. Moreover, the chlorophyll content may be protected probably because of the high antioxidant enzyme activities that increased with sitosterol and prevented degradation of leaf chlorophyll (Sevengor et al., 2011). The results showed that sitosterol could stabilize the integrality of chloroplast membrane and protect the chloroplasts from salt stress. Moreover, application of sitosterol alleviated the damage effects of salt stress on photosynthetic pigment contents by increasing the MSI (Table 1) compared with those of the reference controls. In agreement with these results, Hassanein et al. (2012) found that sitosterol caused a significant increase in photosynthetic pigment contents of salt stressed faba bean plants. In this regard, Senthil-Kumar et al. (2013) reported that sitosterol may have a role in abiotic stress tolerance by enhancing membrane and chlorophyll stability.

Soluble sugar contents

The effects of salinity and sitosterol on total soluble sugars content of V. faba plants are shown in (Fig. 2). Data in the figure showed that total soluble sugars were markedly decreased with increasing levels of NaCl. The lowest value of soluble sugars was recorded at the highest level of salinity stress compared with control plants. In this regard, the 200 mM NaCl resulted in a decrease of about 42% in the soluble sugar content as compared with unstressed plants. Such inhibition in soluble sugar accumulation was recorded by other authors (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 sitosterol stimulated the accumulation of sugars in salt-treated V. faba plants and the inhibitory effects of salt stress were partially alleviated. In this connection, Abd El-Wahed (2001) found that treatment of maize with sitosterol 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 sitosterol 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 sitosterol of carbohydrate biosynthesis, especially soluble sugars that are considered to be the principle organic osmotica in a number of glycophytes subjected to saline conditions (Hassanein et al., 2012), highlight another possible mechanism by which sitosterol plays a positive role in alleviation of the harmful effects of salt stress.

The present study shows that with sitosterol, the leaves fill up more soluble sugar and proline. The increasing of carbohydrate is a signal for water deficiency tolerance. The high carbohydrate concentration with its role to reduce water potential helps to prevent oxidative losses and protein structure maintenance during

water shortage. Also carbohydrates play a molecule role for sugar responsible genes that give different physiological response like defensive response and cellular expansion (Simaei et al. 2012).

Proline content

Data recorded in Fig (3) show that NaCl application caused a significant increase in free proline contents, while with sitosterol the free proline contents of plant leaves markedly decreased. It is clear that proline content increased with increasing salinity level up to 100 mM of NaCl then, tended to decrease at 200 mM of NaCl. At 100 mM of NaCl proline content increased in sitosterol untreated plants by about 51% as compared to 0 mM NaCl control treatment. It is obvious also that sitosterol treatment decreased proline accumulation particularly when plants were not under salt stress. While at salt stress conditions, sitosterol seemed to enhance proline accumulation.

The accumulation of proline concomitant with increasing salinity in Faba bean plants was in agreement with the results obtained by Rahimi et al. (2012), who 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 osmo-protectant 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 (Ben Ahmed et al., 2010). In addition, proline accumulation was reported to serve as a nitrogen storage compound and protect cellular structure (Rahimi et al., 2012). It is also evident from the present study that the level of proline increased in V. faba plants treated with salinity and decreased with sitosterol treatment. This finding might be explained by the fact that sitosterol enhances the biosynthesis of other amino acids and their incorporation into protein.

Antioxidant enzyme activities

The results presented in Table (4) show the effect of sitosterol on the activities of the antioxidant enzymes SOD, CAT, POD, APX and GR in V. Faba plants at the vegetative stage. The activities of enzymes SOD, CAT, POD and APX showed progressive increase with increasing salinity level, whereas the activity of GR significantly decreased with increasing NaCl concentration, compared with those of the non-salt stressed plants. Adding sitosterol had significant effects on the activity of all enzymes under salt stress.

It is well known that salinity stress causes generation of excessive reactive oxygen species (ROS), which leads to cell toxicity, membrane dysfunction and cell death (Chookhampaeng, 2011). Plants have developed enzymatic and nonenzymatic mechanism to scavenge ROS (Hassanein et al., 2012). Among the active oxygen species superoxide is converted by SOD enzyme to H2O2, which is further scavenged by CAT and APX. Over-expression of the APX gene in plants has showed improvement in protection against oxidative stress (Yasar et al., 2008). In
V. Faba plants salt stress 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 and chloroplastic SOD. Increased activity of these antioxidant enzymes is considered to be a salt-tolerance mechanism in most plants.

The present results showed that salt stress caused a decrease in GR activity. GR deactivation by salt stress may be a result of prevention of new enzyme synthesis (Liang et al., 2006). GR activates the glutathione-ascorbate cycle and converts GSSG to reduced glutathione (GSH) (Vega et al., 2003). In addition, GR regulates GSH/GSSG ratio and supplies GSH for GPX and DHAR, which convert H2O2 to H2O and reduce oxidized ascorbate, respectively. The changes in GR activity (Table 5) and lipid peroxidation (LP), as indicated by the accumulation of MDA (Table 1), in V. faba plants subjected to different levels of salinity and silicon are recorded. The GR activity gradually decreased with increasing NaCl concentration, whereas a gradual increase in lipid peroxidation was observed, compared with those of non-salt-stressed plants. The maximum reduction in GR activity (43% of that of the controls) and the maximum increase in MDA (LP) content (77.7 of that of the controls) were detected in plants treated with 200 mM NaCl.

Application of sitosterol 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 sitosterol 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 Vardhini and Rao (2003) and might be an indicator of removal of stressful conditions by brassinosteroids. 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 Bor et al. (2003), who reported an

increase in lipid peroxidation in sugar beet leaves during salt stress. However, 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), 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 sitosterol treatment, which reinforced the suggestion that sitosterol treatment can ameliorate the stressful condition by increasing the stability of membranes in V. faba.

Protein patterns

Changes of protein patterns have been analyzed in leaves of pepper plants (Fig. 4) and Table (5), in order to follow any possible alterations in gene expression in plants subjected to different levels of salt stress in the absence or presence of sitosterol (ST) comparing with non-salt stressed control. It is clear that salt stress and ST treated plants induced variations in the appearance of new protein bands and in disappearance of other bands with different high molecular weights, whereas no changes in protein patterns with low molecular weights were observed. The analysis of protein patterns indicates the following facts: 1) in non-stressed plants; application of ST did not change the pattern of protein bands, while 2) In salt-stressed plants; ST treatment induced the appearance of two new polypeptides (73 and 76 kDa). It appears clearly that ST treatment at 50, 100 and 200 mM NaCl enhanced the formation of a 73 and 76-kDa proteins. In contrast, synthesis of these proteins was negatively affected by the absence of NaCl and by salt stress without ST treatment as shown in panel (a).

The new bands of high molecular weight proteins in salt stressed plants treated with ST might be due to de novo synthesis of these proteins (Gopala Roa et al., 1987). These new proteins may have a specific function to protect pepper plants from further dehydration damage and considered as a defense mechanism to salt stress. Salt stress induced-polypeptides have been observed in many studies and are assumed to play a role in salt stress tolerance (Jiang and Huang, 2002).

Disappearance of certain polypeptides in salt-stressed plants in the absence of ST may be related to increase the hydrolyzing enzyme RNAase activity (Kong-ngern et al, 2005). The effect of ST was clear, suggesting an interaction between the protein synthesis and ST.

It is also clear that the high salt stress treatment (200 mM) did not affect the accumulation of the protein subunits between 25 and 38 KDa (panel a), however, these subunits, particularly that found at 38 KDa, were so condensed when salt stressed plants were treated with ST (Panel b). These data suggest that accumulation of the low MW subunits proteins (between 37- 36 KDa) was insensitive to salt stress, therefore they appear either under salt unstressed or salt stressed samples. Nevertheless, salt stress might have accounted for the delayed onset of high MW protein subunits, relative to the onset of the low MW subunits, under water stress in the absence of ST treatment, therefore the 73 and 76 KDa subunits were formed in ST-treated samples even though they were under severe water stress condition. These results were consistent with Samarah et al. (2006), who reported that, soybean seeds produced under stress had a
variation in β-subunit of the β-conglycinin, probably because of degradation of proteins in the shriveled seeds produced under stress.

Yield and yield components

Fruit setting, total fruit yield and fresh and dry weights of pepper fruit decreased with increasing level of salinity stress (Table 6). The high salt stress (2000 ppm) reduced fruit set%, number of fruits/plant, fresh weight of fruits/plant and dry weight of fruits /plant by about 50%, 70%, 71% and 65%, respectively, as compared to control plants. On the other side, applied ST increased fruit setting, fruit yield, and fresh and dry weights of pepper fruits compared to salt-stressed plants without ST treatment. These increases were observed at all levels of salinity stress including control plants. It seems that ST counteracted the negative effects of salt stress on the yield of pepper plants.

It seems that increasing salinity decreased economic of fruit yield this decrease in fruit yield could be attributed to the decreased number of perfect flowers and fruit set and the increased production of imperfect fruits as it was reported by Grattan et al. (2002). Furthermore a reduction in leaf area reported in the present study (Table 1) might result in reduction in the supply of carbon assimilate due to a decrease in the net photosynthetic rate and biomass accumulation (Sakr et al., 2007). According to the data recorded in this investigation, it was shown that salinity stress decreased many parameters including leaf number, leaf area, dry matter, photosynthetic pigments, and sugar content all of which will ultimately decrease the yield of pepper plants. While ST treatment alleviate the harmful effect of salt stress on yield components and improved the yield parameters due to its positive effect on growth parameters as shown in Table (1).

Table 1. Effect of sitosterol on growth parameters of pepper plants grown under different levels of salinity stress.

Salt (mM)	ST treat.	Plant height (cm)	No. of leaves /plant	Leaf area (cm^2)/plant	Shoot d.wt (g)
00	- ST	43.23±2.11	48.52±2.34	1533±7.45	14.63±0.78
	+ ST	52.34±2.45	65.24±2.36	1727±7.62	18.42±0.75
50	- ST	40.25±2.33	43.23±2.28	1266±6.24	10.55±0.63
	+ ST	42.42±2.24	52.62±2.27	1575±6.45	13.54±0.72
100	- ST	36.54±2.15	38.25±2.11	989±5.34	8.96±0.55
	+ ST	38.53±2.18	45.87±2.18	1214±6.36	10.52±0.66
200	- ST	32.47±2.23	30.54±2.23	677±4.23	6.44±0.44
	+ ST	34.24±2.15	36.28±2.15	856±4.54	8.82±0.55
LSD (5%)		4.22	5.52	145.5	2.65

Each value represents the mean of 3 replicates, – ST means no sitosterol application and + ST means sitosterol application.

Table 2. Effect of sitosterol on electrode leakage (EL), lipid peroxidation (LP) and membrane stability index (MSI) of pepper plants grown under different levels of salinity stress.

Salt (mM)	ST treat	Electrolete leakage (EL %)	Lipid peroxidation (MDA µg/g fwt)	Membrane stability index (MSI %)
00	-ST	12.64±1.22	07.11±0.23	63.56±2.34
	+ST	10.55±1.45	05.62±0.18	86.45±2.56
50	-ST	14.83±1.54	08.24±0.16	62.85±2.76
	+ST	13.25±1.66	06.12±0.22	80.14±2.32
100	-ST	18.52±0.89	10.25±0.28	54.54±2.18
	+ST	16.42±1.11	08.42±0.18	75.55±2.06
200	-ST	20.45±1.23	13.68±0.26	43.32±2.11
	+ST	18.56±1.45	12.11±0.24	65.06±2.33
LSD (5%)		2.25	2.04	4.85

Each value represents the mean of 3 replicates, – ST means no sitosterol application and + ST means sitosterol application.

Table 3. Effect of sitosterol on photosynthetic pigments of pepper plants grown under different levels of salinity stress.

Salt (mM)	ST treat	Chl. a (mg/g fwt)	Chl. b (mg/g fwt)	Carotenoids (mg/g fwt)
00	-ST	1.25±0.23	1.03±0.22	0.65±0.11
	+ST	1.67±0.27	1.24±0.23	0.82±0.12
50	-ST	1.34±0.22	0.85±0.24	0.68±0.14
	+ST	1.82±0.19	1.15±0.23	0.77±0.11
100	-ST	1.02±0.25	0.68±0.27	0.55±0.16
	+ST	1.34±0.26	0.86±0.24	0.64±0.15
200	-ST	0.68±0.25	0.46±0.25	0.32±0.12
	+ST	1.11±0.16	0.62±0.24	0.45±0.14
LSD (5%)		0.32	0.25	0.14

Each value represents the mean of 3 replicates, – ST means no sitosterol application and + ST means sitosterol application.

Table 4. Effects of sitosterol (ST) on antioxidant enzyme activities in faba bean plants grown under different levels of salinity stress.

Salt (mM)	ST treat	SOD (unit g^{-1} FW)	CAT (µM H2O2 g^{-1} FW)	POD (O.D g^{-1} FW min^{-1})	APX (mM ascorbate min^{-1})	GR (µg g^{-1} FW)
00	-ST	22.5±2.11	4.88±0.88	2.65±0.56	0.36±0.11	0.52±0.22
	+ST	28.4±2.23	6.22±0.62	3.34±0.46	0.48±0.12	0.44±0.15
50	-ST	26.6±2.42	4.05±0.53	5.34±0.52	0.46±0.17	0.46±0.17
	+ST	30.2±2.22	5.85±0.36	6.22±0.56	0.52±0.21	0.36±0.15
100	-ST	54.3±2.14	3.16±0.22	6.12±0.45	0.64±0.16	0.32±0.11
	+ST	58.3±2.16	4.74±0.28	7.12±0.58	0.69±0.23	0.25±0.08
200	-ST	42.4±2.12	2.85±0.26	4.33±0.43	0.35±0.15	0.26±0.07
	+ST	48.6±2.27	3.22±0.25	5.15±0.47	0.45±0.20	0.20±0.04
LSD (5%)						

Each value represents the mean of 3 replicates, – ST means no sitosterol application and + ST means sitosterol application.

Table 5. Effect of salinity stress and sitosterol (ST) on protein molecular weights (KDs) of pepper leaves.

NaCl & ST Treatments	Protein MWt (KDs) 7	14	22	24	25	33	34	37	46	48	72	76
0 mM	+	+	+	+	+	+	+	+	+	+		
50 mM	+	+	+	+	+	+	+	+	+	+		
100 mM	+	+	+	+	+	+	+	+	+	+		
200 mM	+	+	+	+	+	+	+	+	+	+		
0 mM+ST	+	+	+	+	+	+	+	+	+	+		
50 mM+ST	+	+	+	+	+	+	+	+	+	+	+	+
100 mM +ST	+	+	+	+	+	+	+	+	+	+	+	+
200 mM +ST	+	+	+	+	+	+	+	+	+	+	+	+

Table 6. Effects of salinity stress and salicylic acid on chemical parameters of pepper plants.

Salt (mM)	ST treat.	Fruit set (%)	Total No. of fruits/plant	F wt. of fruits/plant	D wt. of fruits/plant
00	- ST	11.63±1.22	11.56±1.54	367.8±6.33	26.74±1.33
	+ ST	19.24±2.03	16.45±1.76	535.6±7.45	42.24±1.45
50	- ST	10.46±1.34	7.35±1.34	232.5±5.44	22.52±1.35
	+ ST	16.55±1.67	11.54±1.35	435.3±5.84	31.46±1.45
100	- ST	7.83±1.34	5.81±0.67	204.2±4.34	16.24±1.11
	+ ST	11.52±1.24	8.52±0.63	223.5±4.27	21.76±1.52
200	- ST	5.87±0.34	3.55±0.27	106.6±4.11	9.42±1.23
	+ ST	7.64±0.56	5.63±0.23	180.5±4.15	11.54±1.12
LSD 5%		3.450.28	2.11	56.55	18.82

Each value represents the mean of 3 replicates, – ST means no sitosterol application and + ST means sitosterol application.

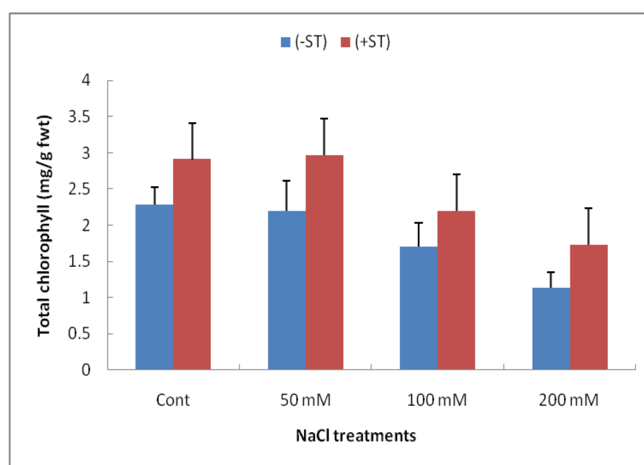

Figure1. Effect of sitosterol (ST) on total chlorophyll of pepper plants grown under different levels of salinity stress.

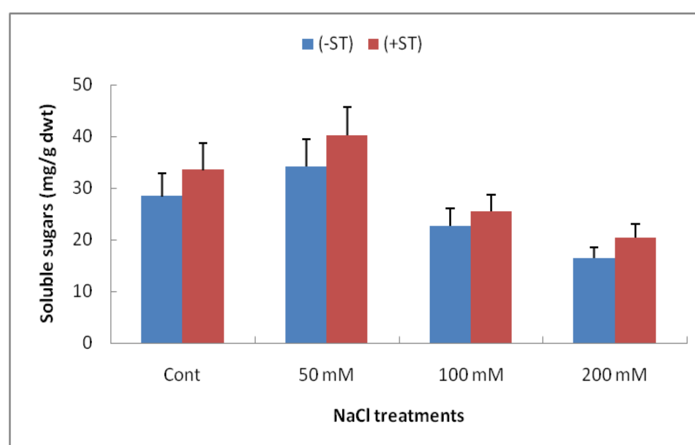

Figure2. Effect of sitosterol (ST) on total soluble sugars of pepper plants grown under different levels of salinity stress.

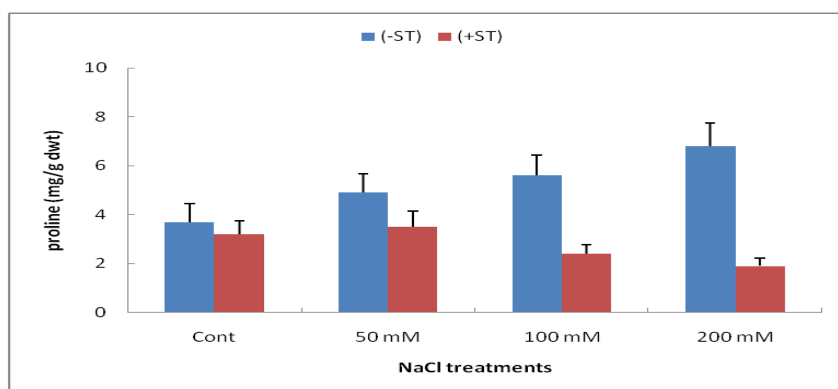

Figure3. Effect of sitosterol (ST) on free proline of pepper plants grown under different levels of salinity stress.

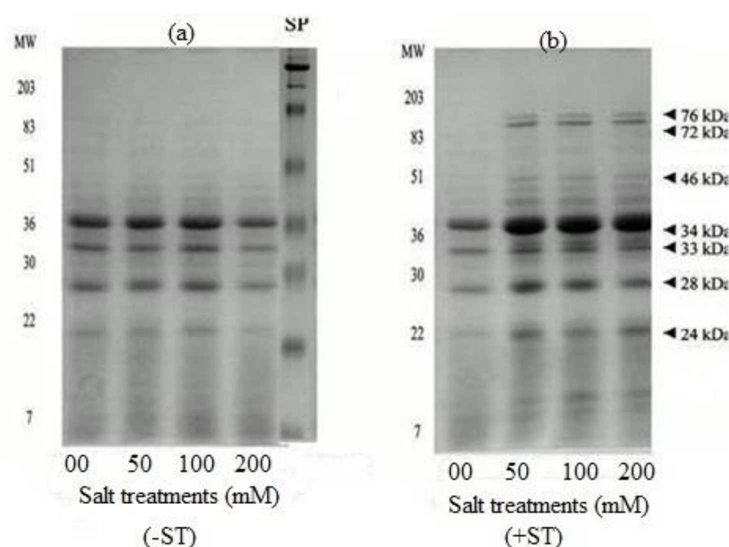

Figure4. Analysis of protein patterns by one-dimensional SDS-PAGE extracted from leaves of pepper (Capsicum annuum L.) plants grown under different levels of salinity stress (0, 50, 100 and 200 mM NaCl) and untreated (-ST)or treated (+ST) with sitosterol (ST). The effect of sitosterol on the approximately 73 and 76 MW protein in the leaves of pepper plants (lanes 2b to 4b) is shown. Lanes (SP) on the left contained marker proteins whose molecular masses (kilodaltons) are shown on the right side of the panels. Sitosterol was used at 200 ppm (b) on pepper seeds grown under different salt concentrations (0, 50, 100 and 200 mM NaCl). The lane on the left side indicates the molecular weights (MW) of standard proteins (SP). The lane on the right is used to specify MW of proteins indicated by arrows.

CONCLUSION

Salt stress even at moderate levels significantly decreased growth and biomass production of pepper plants. High salinity stress decreased the photosynthetic pigments, sugar content and the yield of pepper. The adverse effects of salt stress on the growth and productivity of pepper can be mitigated by foliar spray of sitosterol. However, effectiveness of ST in alleviating the adverse effects of salinity stress was salt-dose dependent. Sitosterol treatment induced augment of enzymatic antioxidant system, reducing oxidative damage (membrane integrity and MDA) in NaCl stressful conditions. The increase in the degree of salt tolerance induced by sitosterol was also reflected in the improvement in the photosynthetic pigments content and consequently the carbohydrate pool and protein pattern in the presence of salinity. Thus, our data provide evidence for the stimulatory effects of sitosterol to induce salt tolerance in faba bean plants.

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