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Mitigation effects of non-enzymatic antioxidants in maize (*Zea mays* L.) plants under salinity stress

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Abstract

The effects of non-enzymatic antioxidative compounds such as ascorbic acid, thiamine HCl and β-carotene were investigated on salt stressed maize plants. The maize plants were sprayed with 100 mg L^{-1} of ascorbic acid, thiamine or β-carotene solutions once a week, up to harvesting of plants. The treatment of NaCl was initiated 25 days after sowing by irrigating the plants with 125 mM NaCl solution. Plants for physiological and biochemical measurements were harvested at the cob formation stage and for yield 90 days after seedling emergence. The results showed that although salt stress reduced the shoot and root dry weights and macro-element contents of maize plants, exogenous application of non-enzymatic antioxidants improved the above-mentioned parameters of maize plants under saline conditions. Shoot and root dry weights increased significantly ($P < 0.05$) by the ascorbic acid treatments. Salt stress enhanced the activities of superoxide dismutase (SOD; EC: 1.15.1.1), peroxidase (POX; EC: 1.11.1.7) and polyphenol oxidase (PPO; EC: 1.10.3.1). Proline content also increased significantly in maize plants in response to NaCl stress. Although the SOD activity increased significantly with ascorbic acid treatment, the POX activity increased considerably with β-carotene application. Of the non-enzymatic antioxidative compounds tested, ascorbic acid was more effective than the others in protecting maize plants from salinity stress. The results of the present study indicate that foliar application of non-enzymatic antioxidative compounds alleviated the detrimental effects of salinity and increased resistance to salinity in the maize plants by improving the antioxidative defense system.

Keywords: Maize, *Zea mays* L. non-enzymatic antioxidants, salinity.
Abbreviations: POX: peroxidase, PPO: polyphenol oxidase, SOD: superoxide dismutase, APX: ascorbate peroxidase, GR: glutathione reductase.

Introduction

Salinity can severely limit crop yield, especially in the most productive areas of the world (Ashraf and Ali, 2008). Salinity-induced reduction in plant growth and yield occurs due to changes in various physiological and biochemical characteristics such as reduced leaf chlorophyll content and photosynthetic capacity, diversion of energy in the processes of osmotic adjustment and ion exclusion, and nutritional imbalance (Munns, 2005). Furthermore, oxidative stress is a common effect of adverse environmental conditions including high soil salinity (Apel and Hirt, 2004). Plants have developed an antioxidant defense system to counteract stress-induced oxidative stress. It is now believed that salt tolerance in most crop plants is associated with a more efficient antioxidant system. The antioxidative system includes both enzymatic and non-enzymatic systems. The non-enzymatic system includes mainly ascorbic acid (vitamin C), ά-tocopherol, carotenoids, and flavonoids, whereas the enzymatic antioxidative system includes superoxide dismutase (SOD), peroxidase (POX), ascorbate peroxidase (APX), glutathione reductase (GR) and polyphenol oxidase (PPO), etc. The function of this antioxidant system is to scavenge the injurious radicals produced during oxidative stress and thus help the plants to survive under such conditions (Mandal et al., 2009). Natural antioxidants occur in all parts of the plant. These antioxidants include carotenoids, vitamins, phenols, flavonoids, dietary glutathione, and endogenous metabolites (Krishnaiah et al., 2011). Plant-derived antioxidants have been shown to function as singlet and triplet oxygen quenchers, free radical scavengers, peroxide decomposers, enzyme inhibitors, and synergists (Lutts et al., 1996). Ascorbic acid is synthesized in higher plants and is one of the key products of D-glucose metabolism. It affects plant growth and development, and plays an important role in the electron transport system (El-Kobisy et al., 2005). Furthermore, it has also been implicated in regulation of cell elongation (Zauberman et al., 1991). Thiamine (vitamin B_1) could serve as a coenzyme in the decarboxylation of α-keto acids, such as pyruvic acid and glutamic acid (Belanger et al., 1995). Thiamine is an important cofactor for the transketolation reactions of the pentose phosphate cycle, which provides pentose phosphate for nucleotide synthesis and to give rise

reduced form of NADP required for various synthetic pathways (Jaleel et al., 2007). Although roots of some plant species can synthesize thiamine, those of other plants cannot synthesize this vitamin (Mittler, 2002). Absorption of thiamine by plant roots has been reported (Mateikene et al., 1988) and leaf applied thiamine can transport in both acropetal and basipetal directions (Mozafar and Oertli, 1992; Mozafar and Oertli, 1993). Despite acting as accessory pigments for photosynthesis, carotenoids have multiple functions in plant metabolism (Taiz and Zeiger, 2010). In particular, they act as potential antioxidants in plants. *β*-carotene has been reported as a key thylakoid membrane-bound antioxidant that can scavenge singlet oxygen species generated from the interaction of P680 and O_2 in photosystem II (Taiz and Zeiger, 2010). Of different carotenoids, β-carotene has been reported to be a bio-stimulant which is thought to be involved in promoting plant growth (Yang et al., 2002). The aim of this work was to assess the effects of non-enzymatic antioxidant compounds such as ascorbic acid, thiamine and β-carotene on some of physiological and growth parameters as well as on the activities of some of antioxidant enzymes in maize plants grown under saline regimes.

Results

Plant dry weight and yield

Salt stress of the root growing medium caused a marked reduction in shoot and root dry weights, plant height, corn seed weight, and stomatal density in maize plants **(Fig 1).** However, exogenous application of all three non-enzymatic antioxidative compounds improved all plant growth and yield parameters in maize plants grown under saline conditions, but the values of these parameters were still lower than those in control plants. There was a promising effect of ascorbic acid and thiamine on improving shoot and root dry weights, plant height, corn seed weight and stomatal density under saline stress, however, the effect of β-carotene was less pronounced on these parameters except root dry weight.

Electrolyte leakage and leaf relative content

Electrolyte leakage, a measure of membrane stability, increased in maize plants under salt stress**.** Exogenous application of non-enzymatic antioxidative compounds were found to be very effective in reducing electrolyte leakage in salt stressed maize plants, and β-carotene being more effective than the other two antioxidant compounds Leaf RWC declined significantly in maize plants due to salinity treatment compared to that in control plants (Fig 2). However, foliar application of non-enzymatic antioxidative compounds significantly improved RWC in salt stressed maize plants and more improvement being with thiamine and β-carotene. Leaf free proline content in the NaCl-stressed plants was higher than that in control plants. However, foliar application of all three non-enzymatic antioxidative compounds to salinized plants reduced the levels of proline compared with those in salt stressed maize plants receiving no exogenous treatment.

Chlorophyll contents

Chlorophyll (chl *a* and chl *b*) and carotenoid contents decreased significantly in maize plants grown under NaCl stress. However, exogenously applied thiamine increased chl *a* and chl *b* contents, but did not affect significantly carotenoid contents in salt stressed maize plants compared to those of salt-stressed plants receiving no treatment of non-enzymatic antioxidative compounds (Fig 3). β-carotenoid application decreased chl *a*, chl *b*, and total chlorophyll contents in salt stressed maize plants. Similarly, exogenously applied ascorbic acid decreased chl *b* and carotenoid contents in maize plants grown under saline stress.

Enzyme activities

The activities of SOD, POX and PPO increased in the leaves of maize plants exposed to saline regime. It is noteworthy that POX activity was more stimulated by NaCl compared with the other antioxidative enzymes. Exogenously applied ascorbic acid significantly improved the activity of SOD, but decreased those of POX and PPO in salt stressed maize plants. β-carotenoid application markedly improved the activity of PPO, but decreased those of SOD and POX in salt stressed maize plants. However, thiamine application decreased the activities of SOD and POX in salt stressed maize plants, whereas it did not alter that of PPO.

Discussion

Salt stress caused a marked reduction in all growth parameters measured in maize plants exposed to saline stress. The salt-induced decrease in growth could be due to salt-induced osmotic and ionic effects on plants (Ghoulam et al., 2002; Munns, 2002). Of the three different biomolecules applied to maize plants, ascorbic acid was found to be very effective in promoting growth of maize plants under saline stress. AsA-induced increase in growth of maize plants under saline conditions may have been due to its role in increasing cell division and/or cell enlargement (Athar et al., 2008). For example, AsA has been reported to accelerate cell division and cell enlargement in different plants such as onion (Cabo et al., 1996), pea (Citterio et al., 1994), and wheat (Athar et al., 2008). It has also been observed that exogenous application of AsA can also counteract salt-induced growth inhibition in plants, e.g., wheat (Al-Hakimi and Hamada, 2001), and sunflower (Sayed and Gadallah, 2002). Athar et al. (2008) observed that AsA applied at 100 mg L^{-1} improved growth of a salt tolerant cultivar of wheat under saline conditions and this AsA-induced enhancement of growth was found to be associated with enhanced photosynthetic efficiency. Similar growth promoting effect of AsA was also observed in chickpea (Beltagi, 2008).
Thiamine, vitamin B1, serves as a precursor of thiamine diphosphate, which acts as a potential coenzyme in many key metabolic pathways, including plant pigment biosynthesis (Friedrich, 1987). However in the present study, thiamine improved growth in maize plants grown under saline stress. These results are analogous to what has been earlier observed in sunflower by Sayed and Gadallah (2002) who showed that root or shoot applied thiamine improved growth, which was found to associated with thiamine-induced reduced membrane injury and increased leaf RWC, chlorophyll content, soluble sugars and total free amino acids. Tunc-Ozdemir et al. (2009) have shown that thiamine application confers enhanced tolerance to oxidative stress in *Arabidopsis* caused by multiple stresses including salinity stress. The thiamine-induced tolerance to

Table 1. The effects of non-enzymatic antioxidants on macro-nutrient contents (mmol kg^{-1} d.wt) in shoot and roots.

Shoot						Root				
Treatments	Na	Ca	K	P	Na:K	Na	Ca	K	P	Na:K
C	121d	245b	587c	67b	0.206 d	217d	137c	700a	44a	0.31b
S	743a	147d	484e	48c	1.535 a	1670b	112d	105b	32b	15.9a
S+AsA	508c	270a	705a	67b	0.720 c	1280c	117d	74c	38ab	17.3a
S+BC	513c	198c	694b	124a	0.739 c	1236c	152b	67c	41ab	18.4a
S+Thi	569b	120e	523d	73b	1.087 b	1857a	197a	112b	44a	16.6a

Different letters in each column represent significant differences at $P < 0.05$, based on LSD test. C: Control; S: 125 mM NaCl; AsA, BC and Thi: 100 mg L^{-1} ascorbic acid, β-carotene, and thiamine sprayed foliar, respectively.

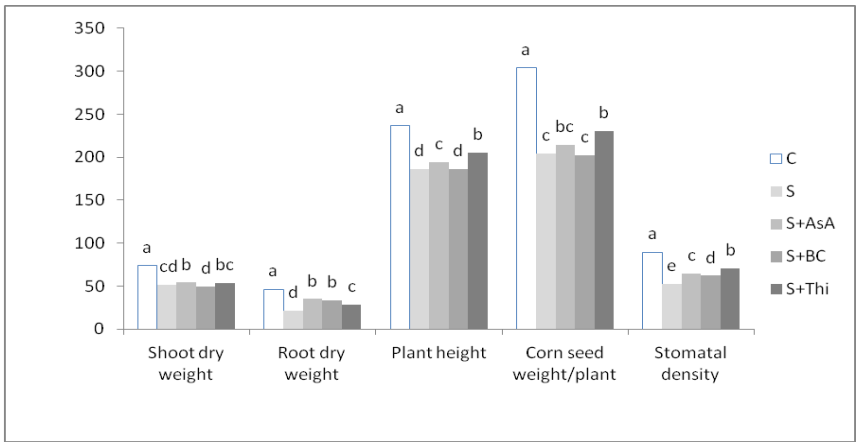

Fig 1. The effects of non-enzymatic antioxidants on plant growth parameters in NaCl stressed maize plants (Different letters on bars within each variable show significant difference at $P < 0.05$) Shoot DW, root DW and corn seed weight (g), plant height (cm), stomatal density (units mm^{-2}) C: Control; S: 125 mM NaCl; AsA, BC and Thi: 100 mg L^{-1} ascorbic acid, β-carotene, and -thiamine sprayed foliarly, respectively.

oxidative stress was found to be associated with reduced generation of ROS in plants which was confirmed by impaired protein carbonylation and H_2O_2 accumulation. Salinity stress decreased chlorophyll *a* and *b* as well as carotenoid contents in maize plants subjected to saline stress in the present study. The decrease in chlorophyll content may have been due to the formation of proteolytic enzymes such as chlorophyllase which is responsible for chlorophyll degradation (Zhao et al., 2006). The most efficient protocol of appraising chloroplast senescence is the determination of chlorophyll degradation, and chlorophyll loss is reported to be due to mainly rapid and considerable accumulation of H_2O_2, a potential ROS. Lipid peroxidation of chloroplast membranes could also partially lead to loss of chlorophyll. Furthermore, salinity stress is known to stimulate the activity of chlorophyll degrading enzyme, chlorophyllase, and it also adversely affects the *de-novo* synthesis of proteins, particularly those which bind chlorophyll molecules (Jaleel et al., 2007). In the present investigation, application of AsA or BC caused either no effect on pigment accumulation (chlorophyll and carotenoids) or even in some cases they caused reduction in pigment concentration. These results are similar to those of Farouk (2011) in which a significant decrease in chlorophyll content has been observed in salt stressed wheat plants due to exogenously applied ascorbic acid. However, in contrast, in many reports an improvement in photosynthetic pigments has been reported due to exogenous application of thiamine or ascorbic acid, e.g., in pumpkin (Proebsting et al., 1990), *Brassica campestris* (Khan et al., 2010) and *Cicer arietinum* (Beltagi, 2008). β-carotene has been reported to play a significant role in the assembly and stability of the D1 protein during senescence caused by osmotic stress in clusterbean cotyledons (Deo and Biswal, 2001). Markgraf and Oelmueller (1991) showed a mandatory role of β-carotene in the assembly of photosystem II in the greening of etiolated tissue. However, exogenous application of this biomolecule caused considerable reduction in photosynthetic pigments in salt stressed maize plants in the present investigation, which could not be explained in view of the promising role of this compound in the biological processes described above. Furthermore, Yang et al. (2002) have shown a marked increase in carotenoid contents in rice due to exogenous application of β-carotene. Membrane stability index, estimated as electrolyte leakage in the maize plants, decreased under salt stress in the present investigation**.** Electrolyte leakage is usually used as an indicator of membrane injuries in salt-treated plants (Mandhania et al., 2006). Cell membrane stability is reported to be associated with salt tolerance in plants (Dionisio-Sese and Tobita, 1998). However, exogenous application of non-enzymatic antioxidative compounds reduced electrolyte leakage in salt stressed maize plants. Our data for electrolyte leakage agree with those of Dolatabadian and Jouneghani (2009) in bean plants who showed that ascorbic acid treatment prevented lipid peroxidation and decreased production of malondialdehyde, a final product of peroxidation of membrane lipids. Their data provide a strong support to the hypothesis that AsA reduces the harmful effects of salinity and then improves membrane stability and hence plant salt tolerance. Moreover, Blokhin et al. (2003) reported that one of the main cellular components susceptible to damage by free radicals is lipids (peroxidation of unsaturated fatty acids of membranes) which consequently change membrane properties and functions. Leaf RWC declined significantly due to salinity treatment compared

Table 2. The effects of non-enzymatic antioxidants on activities of some key enzymes of oxidative defense system (Unit/mg protein).

Treatments	SOD Activity	PPO Activity	POX Activity
C	11.00c	0.049c	24.23d
S	14.28b	0.079a	54.90b
S+AsA	18.95a	0.048c	30.68c
S+BC	12.07c	0.051c	75.17a
S+Thi	13.10c	0.060b	53.55b

Different letters in each column represent significant differences at $P < 0.05$, based on LSD test. C: Control; S: 125 mM NaCl; AsA, BC and Thi: 100 mg L^{-1} ascorbic acid, β-carotene, and thiamine sprayed foliarly respectively.

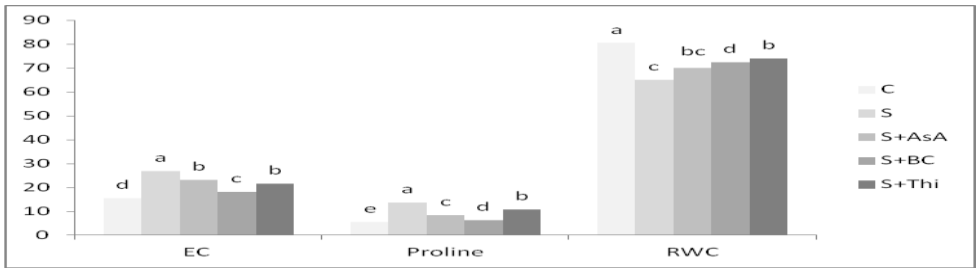

Fig 2. The effects of non-enzymatic antioxidants on electrical conductivity (EC; %), free proline (μmoles g^{-1} FW), and relative water content (RWC %) in NaCl stressed maize plants C: Control; S: 125 mM NaCl; AsA, BC and Thi: 100 mg L^{-1} ascorbic acid, β-carotene, and -thiamine sprayed foliar, respectively.

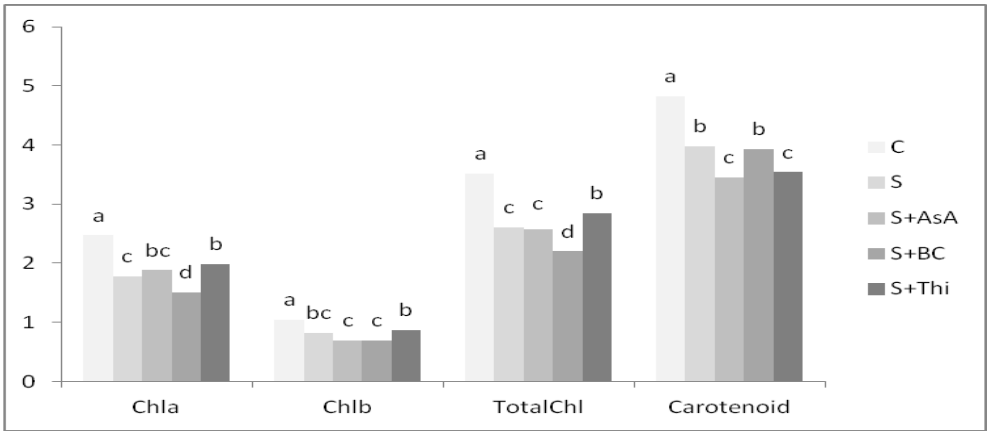

Fig 3. The effects of non-enzymatic antioxidants on chlorophyll (mg g^{-1} FW) and carotenoid (mg g^{-1} FW) contents in NaCl stressed maize plants (Different letters on bars within each variable show significant difference at $P < 0.05$) C: Control; S: 125 mM NaCl; AsA, BC and Thi: 100 mg L^{-1} ascorbic acid, β-carotene, and -thiamine sprayed foliar, respectively.

to control treatment in maize plants. This could be due to a reduction of water supply to the leaves (Tuna et al., 2008). However, foliar application of non-enzymatic antioxidative compounds significantly improved RWC in salt stressed maize plants and more improvement being with thiamine and β-carotene. The maize plants treated with non-enzymatic antioxidative compounds had higher chlorophyll and RWC and they produced more biomass than the non-treated plants. These results are in agreement with those of Proebsting et al. (1990). This shows that thiamine can alleviate the adverse effects of salinity stress by acting as a growth factor to enhance shoot and root growth under saline conditions. Leaf free proline content in NaCl-stressed plants was higher than that in control plants. However, foliar application of non-enzymatic antioxidative compounds to salinized maize plants decreased the levels of proline compared with salt stressed plants receiving no exogenous chemical treatment (Fig 2). The effect of foliar application of β-carotene in reducing the proline content was more striking as compared to those of other compounds. When plants are exposed to a high concentration of salt, some tolerate salinity stress by producing organic solutes in the cytoplasm to lower the osmotic potential. Proline has been proposed to act as a compatible solute that adjusts osmotic potential in the cytoplasm (Ashraf and Foolad, 2007). Proline also participates in radical detoxification and enzyme protection (Gadallah, 1999). In addition, Al-Hakimi and Hamada (2001) reported that grain soaking in AsA and thiamine could counteract the adverse effects of NaCl salinity on wheat seedlings by suppressing salt stress-induced accumulation of proline. Moreover, in the present study, foliar application of AsA decreased proline accumulation in salt stressed maize plants compared to the stressed plants receiving no exogenous treatment. It is well

established that the concentration of proline, which makes up to 80% of the total amino acid pool, increases up to 100 times the normal level in many plants when subjected to stress conditions (Dolatabadian et al., 2009). In the current study, we found that salt stress increased the leaf proline content, which might have contributed to osmotic adjustment and allowed the plants to maintain turgor pressure thereby enabling them to adapt to salinity. It is possible that application of AsA scavenged ROS and prevented biosynthesis of extra proline. The activities of SOD, POX and PPO increased in the leaves of maize plants under saline stress**.** It is noteworthy that POX activity was more stimulated by NaCl as compared to the other enzymes. It is now known that salinity-induced oxidative stress may be overcome by up-regulation of antioxidative enzymes (Ashraf and Ali, 2008). In cowpea, the SOD activity was reported to remain unchanged, but the POX activity increased (Cavalcanti et al, 2004; Cavalcanti et al, 2007). The salinity-induced enhanced activity of antioxidant enzymes has been reported in different studies, e.g., wheat shoot (Meneguzzo et al., 1999; Sairam and Srivastava, 2002), maize (Tuna et al., 2008), and pea (Hernandez et al., 1999). POX and PPO are two major enzymes which actively take part in the oxidation of phenolic compounds (Dolatabadian et al., 2009). The increased PPO activity in maize plants might reduce the phenol accumulation in plants under stress. Increased PPO activity was also reported by Demir and Kocaliskan (2001) in salt stressed bean seedlings. In another study, Shalata and Neumann (2001) reported that root-applied AsA improved the growth of tomato seedlings under saline conditions by specifically improving their antioxidant capacity. This view was further supported by Athar et al. (2009) that exogenous application of AsA caused differential uptake of AsA, which triggered CAT-POD-SOD antioxidative system activation, and maintained ion and water homeostasis thereby protecting photosynthetic machinery of wheat against salt-induced oxidative stress. AsA is a key component of the ascorbate–glutathione cycle and plays a protective role against ROS (Noctor and Foyer, 1998). High level of endogenous ascorbate is essential to maintain the antioxidant system that protects plants from oxidative damage caused by different types of stresses including salt stress. It is well evident that plant cells are strongly redox-buffered due to very large quantities of the water soluble antioxidants including ascorbate (Athar et al., 2009; Hartmann et al., 2003). Thus, it is possible that enhanced endogenous AsA due to root-applied AsA might have protected plants from salt-induced oxidative damage by controlling cellular redox state (Athar et al., 2008). It seems possible that oxido-reductases, POX and PPO may play an important role in the oxidative defense system against salt stress. The enhanced activities of SOD, POX and PPO under NaCl stress, probably come from an increased capacity for oxygen radical scavenging and maintenance of cellular membranes. This indicates the relationship between salt tolerance and antioxidant defense system (Agarwal and Pandey, 2004). Salinity significantly increased Na^+ concentration in maize plants in the present study. Similar results were reported in sugar beet cultivars (Ghoulam et al., 2002), and rice (Lutts et al., 1996). This accumulation of Na^+ ions might be involved in osmotic adjustment in plants subjected to saline medium. Salinity stress reduced K^+, Ca^{2+} and P content in the leaves of maize plants. Like in most glycophytic crops, saline conditions can induce K deficiency in wheat (Al-Hakimi and Hamada, 2001) and maize plants (Botella et al., 1997). In addition, accumulation of K^+ and Ca^{2+} in the wheat leaves and roots was significantly reduced due to salt stress. However, application of 100 mg L^{-1} AsA enhanced the accumulation of K^+ and Ca^{2+} in the leaves and roots of salt-stressed plants (Athar et al., 2008). From these reports it is suggested that AsA-induced ionic changes might have triggered the antioxidant system. Thus, AsA-induced enhanced salt tolerance in wheat plants was due to having a better antioxidant system for the effective removal of ROS from plants and maintenance of ion homeostasis (Athar et al., 2008; Mittler, 2002). The decline in tissue K^+ concentration may have resulted from the direct competition between K^+ and Na^+ at sites of uptake at the plasmalemma, an effect of Na^+ on K^+ transport into the xylem, and/or a Na^+-increased K^+ efflux from the root (He and Cramer, 1993). However, some plants possess the ability to maintain adequate K^+ absorption in the presence of high Na^+ concentrations in the growth medium, thus maintaining a favorable K^+-Na^+ ratio in the leaves (Benes et al., 1996; Hussain et al., 2003). Salt stressed maize plants accumulated significantly lower amount of Na^+ and higher that of K^+, Ca^{2+} and P upon foliar applications of AsA, BC or Thi as compared to those of salt stressed plants receiving no exogenous treatment. Na^+:K^+ ratio in the leaves and roots increased significantly after the exposure of plants to NaCl stress, being significantly more in roots, but foliar application of AsA, BC or Thi to salinized plants significantly decreased the leaf Na^+:K^+ ratio, whereas that of root remained unaffectd. As compared to Thi, AsA and BC were found more effective in improving the contents of Ca^{2+} and K^+ and reducing Na^+ in the leaves of salt stressed maize plants. According to Athar et al. (2009), accumulation of K^+ and Ca^{2+} in the leaves and roots of wheat plants decreased significantly due to salt stress. However, application of AsA enhanced the accumulation of K^+ and Ca^{2+} in the leaves and roots of salt stressed plants. In addition, exogenously applied AsA caused a further reduction in leaf K^+/Na^+ ratio in maize plants.

Materials and methods

Plant culture and treatments

The experiment was conducted in a glasshouse in Mugla (Turkey) from the middle of August to the middle of November. Three seeds of cv. DK 647 of maize (*Zea mays* L.) were sown directly in plastic pots each containing 8 kg of peat, perlite and sand (1:1:1, v/v/v). After germination, the pots were thinned to one plant per pot and then grown for 12 weeks under glasshouse conditions at average day/night temperatures of 25/15°C (max./min. temperature of 32/12°C). The pots were covered with a black plastic sheet to protect the roots from light as well as to prevent evaporation. All chemicals used were of analytical grade, and the composition of the nutrient solution used was (in mM): 15 N (in NO_3 form), 1 P, 6 K, 5 Ca, 2 Mg, 2 S, 0.05 Fe, 0.01 Mn, 0.05 B, 0.003 Cu, 0.0008 Zn and 0.001 Mo. The pH of the nutrient solution was adjusted to 6.0 with 0.1 mM KOH during the entire growing period. The volume of the nutrient solution applied to the root zone of the plants ranged from 200 to 1000 ml from August to October each time twice a week depending on plant age. Each treatment was replicated three times and each replicate included 5 pots (i.e. 15 pots per treatment). Two plants per replicate were uprooted carefully from each pot to determine the electrolyte leakage,

chlorophyll content, leaf relative water contents, soluble protein, and leaf mineral nutrients at cob formation stage. The remaining plants were harvested 90 days after seedling emergence to record grain yield. Twenty-five days after germination, different treatments were initiated. Treatments were: (I) control (C): plants receiving nutrient solution only, (II) salinity treatment (S): plants receiving 125 mM NaCl, (III) salinity plus supplementary 100 mg l^{-1} thiamine (S + Thi), (IV) salinity plus supplementary 100 mg l^{-1} ascorbic acid (S + AsA) and (V) salinity plus supplementary 100 mg l^{-1} β-carotene (S + BC). The solutions of Thi, ASA and BC mixed with 0.01 percent Tween-20 ($C_{58}H_{114}O_{26}$) were applied to leaves once a week. The volume of sprays applied ranged from 25 to 50 ml per pot depending on plant age. Control plants were sprayed with an equal amount of water containing only 0.01 percent Tween-20.

Physiological and biochemical determinations

At maturity, three plants per replicate were harvested for determining grain yield. All maize cobs of harvested plants were removed from the stalks, threshed and weighed to record grain weight. The grain moisture content at threshing was about 13%. Membrane stability index (MSI) was assessed as electrolyte leakage described by Lutts et al. (1996), using young leaf discs of nine plants from each treatment (three per replicate). Leaf discs were put in test tubes each containing 10 ml of distilled deionized water. The tubes containing leaf discs were then subjected to 25°C for 24 h and thereafter electrical conductivity (EC) of the solution (C_1) measured with an EC meter. The samples were finally autoclaved at 120°C for 20 min and the final EC (C_2) recorded after cooling the samples at 25°C. MSI was calculated using the following formula:
MSI = (C_1/C_2) X 100. Chlorophyll and carotenoid contents were estimated by extracting 0.05 g of the leaf material in 10 cm^3 dimethylsulfoxide (DMSO) (Hiscox and Israelstam, 1979). Leaf relative water content (RWC) of fully expanded third leaf was measured at noon (13:00 hours) following Barrs and Weatherley (1962). Free proline in leaf tissue (3^{rd} leaf) was determined according to the method described by Bates et al. (1973). Protein content in the leaf extracts was determined following Bradford (1976) using Bovine Serum Albumin V as a standard. For enzyme extractions and determination assays, approximately 1 g of frozen (in -40^{o}C) plant leaf material was homogenized in 50 mM phosphate buffer (pH 7.0) containing 1% polyvinyl pyrrolidine (PVP). The homogenate was filtered and then centrifuged in a refrigerated centrifuge (at 4^{o}C) at 12000×*g* for 15 min, and the supernatant obtained was used as a source of enzymes. All steps in the preparation of enzyme extract were carried out at 4°C. Meanwhile the extract was stored at –40°C until use. The activity of superoxide dismutase (EC: 1.15.1.1) was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium as described by Beauchamp and Fridovich (1971). Peroxidase (EC: 1.11.1.7) activity was determined using the guaiacol oxidation method of Chance and Maehly (1955). Polyphenol oxidase (EC: 1.10.3.1) activity was assayed with 4-methylcatechol as a substrate according to the method of Zauberman et al. (1991). For nutrient analysis and dry weight determinations, shoots and roots of three randomly selected plants per replicate were separated and dried in a forced air oven for two days to determine dry weights. Chemical analyses were carried out on dry weight basis. The dried samples were ground to fine powder using a pestle and mortar and stored in polyethylene bottles. For Na^+, Ca^{2+}, K^+ and P analysis, samples of dried leaves and roots were ashed in a furnace for 6 h at 500°C and then the ash was taken in 5 mL of 2 M hot HCl, filtered into a 50 mL volumetric flask and made up the volume to 50 mL with distilled water. Na^+, Ca^{2+}, K^+ and P contents were determined by Varian model ICP-AES (Chapman and Pratt 1961).

Statistical analysis

Treatments were replicated three times and each replicate contained 5 pots (i.e. 15 pots per treatment). All treatments were placed in a randomized complete block design. Data for all parameters were statistically analyzed using the Statview-ANOVA statistical package. Statistically different groups were compared using an LSD test ($P < 0.05$).

Conclusion

Finally, it can be concluded that amongst the non-enzymatic antioxidative compounds tested, ascorbic acid was more effective than the others, in protecting maize plants against the adverse effects of salinity stress. The results of the present study indicate that foliar-applied non-enzymatic antioxidative compounds alleviated the detrimental effects of salinity and increased resistance to salinity by up-regulating the antioxidative defense system in maize plants.

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