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An Enhancing Effect of Exogenous Mannitol on the Antioxidant Enzyme Activities in Roots of Wheat Under Salt Stress

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Abstract The role of mannitol as an osmoprotectant, a radical scavenger, a stabilizer of protein and membrane structure, and protector of photosynthesis under abiotic stress has already been well described. In this article we show that mannitol applied exogenously to salt-stressed wheat, which normally cannot synthesize mannitol, improved their salt tolerance by enhancing activities of antioxidant enzymes. Wheat seedlings (3 days old) grown in 100 mM mannitol (corresponding to −0.224 MPa) for 24 h were subjected to 100 mM NaCl treatment for 5 days. The effect of exogenously applied mannitol on the salt tolerance of plants in view of growth, lipid peroxidation levels, and activities of antioxidant enzymes in the roots of salt-sensitive wheat (*Triticum aestivum* L. cv. Kızıltan-91) plants with or without mannitol was studied. Although root growth decreased under salt stress, this effect could be alleviated by mannitol pretreatment. Peroxidase (POX) and ascorbate peroxidase (APX) activities increased, whereas superoxide dismutase (SOD), catalase (CAT), and glutathione reductase (GR) activities decreased in Kızıltan-91 under salt stress. However, activities of antioxidant enzymes such as SOD, POX, CAT, APX, and GR increased with mannitol pretreatment under salt stress. Although root tissue extracts of salt-stressed wheat plants exhibited only nine different SOD isozyme bands of which two were identified as Cu/Zn-SOD and Mn-SOD, mannitol treatment caused the appearance of 11 different SOD activity bands. On the other hand, five different POX isozyme bands were determined in all treatments. Enhanced peroxidation of lipid membranes under salt stress conditions was reduced by pretreatment with mannitol. We suggest that exogenous application of mannitol could alleviate salt-induced oxidative damage by enhancing antioxidant enzyme activities in the roots of salt-sensitive Kızıltan-91.

Keywords Antioxidant enzymes · Lipid peroxidation · Mannitol · Root · Salinity · Wheat (*Triticum aestivum* L.)

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Introduction

Salinity of soil or water is one of the most serious factors influencing crop productivity and induces water deficit even in well-watered soils by decreasing the osmotic potential of soil solutes, thus making it difficult for roots to extract water from the surrounding media. Salt stress also exacerbates the effects of other stresses, for example, high light and extreme temperatures. In addition, high salt levels can influence the balance of other ions within cells, leading to ion deficiencies (Marschner 1995). One of the biochemical changes that occurs when plants are subjected to salt stress is the production of reactive oxygen species (ROS) such as the superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH^{\bullet}$). ROS can have a detrimental effect on normal metabolism through oxidative damage to lipids, proteins, and nucleic acids (Mittler 2002). However, many plants that live in saline habitats have evolved several adaptations that confer tolerance to salt and resistance to water deficit. Such plants can prevent salt from entering the root or shoot or they can compartmentalize sodium and chloride within the vacuole and prevent toxic levels of the ions from developing in the cytoplasm. Most halophytes react to environmental stresses with an effective ROS scavenging system involving antioxidant molecules like

carotenoids, ascorbate, glutathione, and tocopherols as well as antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR) (Greenway and Munns 1980; O'Neill 1983). Recent studies have demonstrated that the activities of these antioxidant enzymes and the levels of antioxidant molecules can be increased under various environmental stresses (Hernandez and others 2000; Sekmen and others 2007).

Most halophytes can also accumulate compatible solutes. Common examples of this type of solute include betains and polyols such as mannitol (Bohnert and others 1995). Two obvious functions of compatible solutes are osmotic adjustment and an ability to act as a ROS scavenger.

Among polyols, mannitol is normally synthesized as a primary photosynthetic product and can be metabolized by some plant species (Trip and others 1964; Tholakabavi and others 1994; Stoop and others 1996; Noiraud and others 2001; Conde and others 2007). However, many important agronomical crops such as wheat and tobacco cannot produce mannitol. Therefore, it has long been a goal to introduce a mannitol synthesis mechanism and an increased stress tolerance in these plants. To date, most studies concerned with mannitol have focused on its physiologic role, its utilization by plants, its biosynthesis pathway, its effects on plants as an osmolyte, and its role as a radical scavenger (Smirnoff and Cumbes 1989; Shen and others 1997; Abebe and others 2003). Recent studies have shown improved tolerance of mannitol-producing transformant plants against water and salt stress. In these plants, mannitol biosynthesis and accumulation increased while its catabolism decreased under stress conditions (Everard and others 1993; Williamson and others 2002; Abebe and others 2003; Sickler and others 2007). However, the function of exogenously applied mannitol in the antioxidant enzyme activities of wheat plants, which cannot produce mannitol, has not been evaluated under salt stress. Therefore, in this study we exposed wheat plants to salt stress and investigated whether exogenous application of mannitol could improve tolerance to salt stress by enhancing the activities of antioxidant enzymes in a salt-sensitive wheat cv. Kızıltan-91.

Material and Methods

Plant Material

Seeds of wheat (*Triticum aestivum* L. cv. Kızıltan-91) plants were obtained from the Aegean Agricultural Research Institute, Menemen, Izmir, Turkey. Seeds were surface sterilized with 0.5% sodium hypochloride solution on a magnetic stirrer for 5 min and thoroughly washed with deionized water. Seeds were soaked in sterile deionized water at 25°C for 2 h and then transferred to two sheets of sterile filter paper. Seeds were placed in plastic trays for germination at 25°C for 72 h in the dark. Germinated seeds were sown in holes of styrofoam boards in half-strength Hoagland solution and grown hydroponically in the growth room for 3 days under fluorescent and incandescent lights with a PAR of 350 μmol m^{-2} s^{-1}. The temperature of the growth room was maintained at 25 ± 2°C and daytime humidity was between 60% and 70%. Preliminary studies using various concentrations of mannitol (25–150 mM) and two different times (24 or 72 h) have shown that 100 mM mannitol for 24 h was closer to the optimum combination to acclimate the seedlings. For mannitol + NaCl treatment, one group of wheat seedlings (3 days old) was grown in 100 mM mannitol (corresponding to −0.224 MPa) for 24 h and then transferred from the media with mannitol to media with 100 mM NaCl (no mannitol) for 5 days. The mannitol treatment group was treated with 100 mM mannitol alone for 24 h and was not subjected to salinity. The NaCl treatment group of plants was exposed to 100 mM NaCl alone for 5 days.The last group of wheat seedlings served as a control (treated with neither mannitol nor NaCl). Roots of wheat seedlings were harvested on days 0 and 5 of salt treatment and then stored at −20°C until analyses.

Growth Parameters

After 5 days of NaCl treatment, 20 plants from each group were divided into separate shoot and root fractions. The lengths of roots were measured. For determination of dry weight (DW), roots were dried in a forced-draft oven at 70°C for 72 h and then weighed.

Mannitol Determination

Determination of mannitol was done spectrophotometrically using the modified procedures of Lunn and others (1989). Root samples from each group (0.5 g) were homogenized with ice-cold water acidified to pH 3. The homogenate was centrifuged (5000*g* at 4°C) and the supernatant was used for the determination of mannitol. The reaction mixture contained 500 mM glycine, 500 mM hydrazine sulfate (pH 8.5), 0.2 mM $NAD(P)^+$, and 0.5 unit of mannitol dehydrogenase (EC 1.1.1.67, Sigma, St. Louis, MO). The reaction was started by adding sample. The blank cuvette contained the sample and the reaction mixture given above except for the enzyme, which was replaced by water. The reduction of $NAD(P)^+$ was followed by an increase in absorbance at 340 nm. The mannitol content was calculated according to Beutler (1988).

Lipid Peroxidation

The level of lipid peroxidation in root samples was determined in terms of malondialdehyde (MDA) content according to Madhava Rao and Sresty (2000). MDA content, which is an end-product of lipid peroxidation, was determined using the thiobarbituric acid reaction. Root samples (0.5 g) were homogenized in 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 10,000*g* for 10 min. The supernatant was used for the estimation of MDA content. To a 1-ml aliquot of the supernatant, 4 ml 0.5% thiobarbituric acid (TBA) in 20% trichloroacetic acid (TCA) was added. The mixture was heated at 95°C for 30 min and then cooled in an ice bath. The mixture was then centrifuged at 10,000*g* for 15 min. The absorbance of the supernatant was recorded at 532 nm. Values of nonspecific absorption recorded at 600 nm were subtracted from the values recorded at 532 nm. The MDA content was calculated using its absorption coefficient of 155 $mmol^{-1}$ cm^{-1} and expressed as nmol MDA g^{-1} FW.

Enzyme Extractions and Assays

All operations were performed at 4°C. For protein and enzyme extractions, root samples (0.5 g) were homogenized with 0.05 M sodium phosphate buffer (pH 7.8) containing 1 mM EDTA·Na_2 and 2% (w/v) polyvinylpolypyrrolidone (PVPP). Homogenates were centrifuged at 14,000*g* for 40 min at 4°C. The supernatants were used for the determination of protein content and activities of SOD, POX, CAT, APX, and GR enzymes. Total soluble protein contents were determined according to Bradford (1976) by using bovine serum albumin as a standard. All spectrophotometric analyses were conducted on a Shimadzu (UV 1600).

Superoxide dismutase (SOD; EC 1.15.1.1) activity was assayed by its ability to inhibit photochemical reduction of nitrotetrazolium blue chloride (NBT) at 560 nm (Beuchamp and Fridovich 1971). The assays were performed at 25°C. The reaction mixture (3 ml) contained 33 μM NBT, 10 mM L-methionine, 0.66 mM EDTA·Na_2, and 0.0033 mM riboflavin in 0.05 M sodium phosphate buffer (pH 7.8). The reaction was started by adding riboflavin and placing the tubes containing reaction mixture under 300 μmol m^{-2} s^{-1} irradiance at 25°C for 10 min. A complete reaction mixture without enzyme, which gave the maximal color, served as the control. A nonirradiated complete reaction mixture served as a blank. The absorbance was recorded at 560 nm. One unit of SOD enzyme activity was defined as the quantity of enzyme that reduced the absorbance reading of samples to 50% in comparison with tubes lacking enzymes.

Peroxidase (POX; EC 1.11.1.7) activity was determined according to the method of Herzog and Fahimi (1973). The reaction mixture contained 3,3′-diaminobenzidine-tetrahydrochloride dihydrate (DAB) solution containing 0.1% (w/v) gelatine, 150 mM Na-phosphate-citrate buffer (pH 4.4), and 0.6% H_2O_2. A unit of POX activity was defined as μmol ml^{-1} H_2O_2 decomposed min^{-1}.

Catalase (CAT; EC 1.11.1.6) activity was determined according to the method of Bergmeyer (1970), which measures the initial rate of disappearance of H_2O_2 at 240 nm. The reaction mixture contained 0.05 M Na-phosphate buffer (pH 7.0) with 0.1 mM EDTA, distilled water, enzyme, and 3% H_2O_2. Adding H_2O_2 started the reaction and a decrease in absorbance at 240 nm was recorded for 3 min. A unit of CAT activity was defined as 1 μmol H_2O_2 consumed ml^{-1} min^{-1}.

Ascorbate peroxidase (APX; EC 1.11.1.11) activity was determined according to Nakano and Asada (1981). The assay depends on the decrease in absorbance of ascorbic acid at 290 nm because of oxidation of ascorbic acid to monodehydroascorbic acid and dehydroascorbic acid. The enzyme extract was prepared as in the case of SOD except that the extraction buffer contained 1 mM ascorbic acid in addition to other ingredients. The reaction mixture contained 0.05 M Na-phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM EDTA Na_2, 1.2 mM H_2O_2, and 0.1 ml enzyme extract in a final assay volume of 1 ml. The reaction was started with the addition of hydrogen peroxide. Decrease in absorbance for 3 min was measured at 290 nm in an UV-visible spectrophotometer. Enzyme activity was calculated as the concentration of ascorbic acid oxidized min^{-1} mg^{-1} protein by using the molar extinction coefficient ($\varepsilon = 2.8$ mM^{-1} cm^{-1}).

Glutathione reductase (GR; EC 1.6.4.2) activity was determined according to Foyer and Halliwell (1976). The reaction mixture contained 0.025 M Na-phosphate buffer (pH 7.8), 0.5 mM GSSG, 0.12 mM NADPH·Na_4, and 0.1 ml enzyme extract in a final assay volume of 1 ml. After the addition of ascorbate to the mixture, oxidation of NADPH was followed at 340 nm for 3 min and activity was calculated by using the molar extinction coefficient ($\varepsilon = 6.2$ mM^{-1} cm^{-1}) for NADPH. One unit of GR was defined as 1 mM GSSG reduced ml^{-1} min^{-1}.

Electrophoretic SOD and POX Separation

Before loading on the gels, freshly harvested root samples of salt-sensitive Kızıltan-91 were homogenized with 9 mM Tris HCl (pH 6.8) and 13.6% (v/v) glycerol at 4°C. Homogenates were centrifuged at 14,000*g* for 5 min at 4°C and the supernatants were used for determination of enzyme activity. Protein concentration was determined according to Bradford (1976) using bovine serum albumin as a standard. Samples containing equal amounts of protein (40 μg/well) were subjected to nondenaturating

Table 1 Effects of mannitol on root length and root dry weight of Kızıltan-91 on the 0 and 5th days of salt treatment

Values are mean ± SE of six replicates ($n = 6$). Means followed by the same letters are not significantly different; $P < 0.05$

Days	Control	100 mM Mannitol	100 mM NaCl	100 mM Mannitol + NaCl
Root length (cm)				
0 day	$12.0^{a} \pm 0.92$	$11.10^{a} \pm 0.91$		
5th day	$13.9^{b} \pm 1.59$	$13.53^{b} \pm 1.53$	$10.90^{a} \pm 0.53$	$12.88^{b} \pm 0.75$
Dry weight (g)				
0 day	$6.88^{a} \pm 0.3$	$6.26^{a} \pm 0.3$		
5th day	$8.42^{c} \pm 0.5$	$8.49^{c} \pm 0.4$	$7.56^{b} \pm 0.6$	$8.81^{c} \pm 0.9$

polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (1970) except that sodium dodecyl sulfate was omitted.

For the separation of SOD isozymes, samples containing 40 µg protein were subjected to native PAGE in 5% stacking and 12% separating gels under constant current (120 mA) at 4°C. SOD activity was detected by photochemical staining with riboflavin and NBT as described by Beuchamp and Fridovich (1973). The different types of SOD were differentiated by adding inhibitors to staining solution, such as 2 mM KCN to inhibit Cu/Zn-SOD activity and 3 mM H_2O_2 to inhibit Cu/Zn-SOD and Fe-SOD activities as described by Vitória and others (2001). Mn-SOD activity is resistant to both inhibitor treatments.

Electrophoretic POX separation was done according to Seevers and others (1971). For POX separation, samples containing 40 µg protein were subjected to 10% native PAGE under constant current (120 mA). To visualize the band patterns, the gels were incubated in the dark for 30 min at 30°C in 200 mM Na-acetate buffer (pH 5.0) containing 1.3 mM benzidine and 3% hydrogen peroxide. The gels were then stored in 7% acetic acid.

We originally performed four independent replicates of SOD and POX gels.

Statistical Analysis

All analyses were done on a completely randomized design. All data obtained were subjected to one-way analyses of variance (ANOVA) and the mean differences were compared by lowest standard deviations (LSD) test. Each data point was the mean of six replicates ($n = 6$) and comparisons with $P < 0.05$ were considered significantly different. In all the figures, the spread of values is shown as error bars representing standard errors of the means.

Results

Growth Parameters

To investigate the effects of exogenous mannitol in wheat, we measured root growth by root length and dry weight (Table 1). We found that salt stress reduced root length by 22% (Table 1). However, root length was reduced only by 7% in plants pretreated with mannitol under salt stress. Therefore, reduction in root length under salt stress was significantly alleviated by mannitol pretreatment. Similar to the changes in root length, root dry weight was reduced (10%) by salt stress, whereas the root dry weight of plants pretreated with mannitol was not significantly reduced under identical conditions. Thus, we can say that under salt stress conditions, reduction in root dry weight was significantly prevented by mannitol pretreatment.

Mannitol Content

Mannitol was applied to 3-day-old wheat seedlings for 24 h via nutrient solution. Subsequently, the seedlings were transferred from the media with mannitol to media with 100 mM NaCl (no mannitol) for 5 days. At the end of this 5-day period, the mannitol content was measured spectrophotometrically (Table 2). Mannitol treatment induced incorporation of mannitol in roots under salt stress. It was found that mannitol content was the highest (22%) in roots of mannitol + NaCl-treated plants on the 5th day of NaCl treatment. However, no mannitol was detected in control and NaCl-treated Kızıltan-91 plants.

Lipid Peroxidation

Lipid peroxidation levels in roots of wheat plants determined as the content of MDA are given Fig. 1. Salt

Table 2 Mannitol concentration (g mannitol l^{-1} sample solution) in roots of Kızıltan-91 on the 0 and 5th days of salinity

Groups	0 day	5th day
Control	ND	ND
100 mM mannitol	$0.024^{a} \pm 0.001$	$0.052^{b} \pm 0.003$
100 mM NaCl		ND
100 mM NaCl + 100 mM mannitol		$0.067^{c} \pm 0.003$

ND = not detected

Values are mean ± SE of six replicates ($n = 6$). Means followed by the same letters are not significantly different; $P < 0.05$

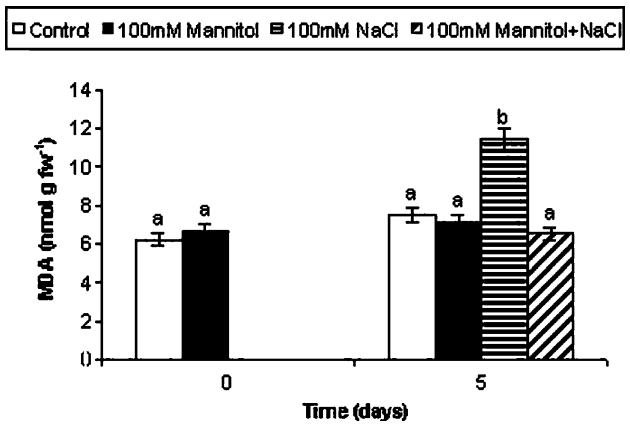

Fig. 1 Effect of exogenous mannitol on the MDA (nmol g^{-1} FW) content in roots of Kızıltan-91 before and after exposure to salt. Values are mean ± SE of six replicates ($n = 6$). Bars with different letters are significantly different at $P < 0.05$

treatment alone caused an induction (53%) in MDA content. However, MDA content was significantly reduced by 43% in mannitol + NaCl-treated plants in comparison to the plants receiving NaCl alone. It is clear that salt stress increased lipid peroxidation in roots of Kızıltan-91 and was significantly alleviated by mannitol pretreatment.

Antioxidant Enzyme Activities

The effects of exogenous mannitol on the antioxidant enzyme activities of SOD, POX, CAT, APX, and GR in the roots of Kızıltan-91 are shown in Fig. 2. Mannitol increased SOD activity under salt stress (Fig. 2a). However, salt treatment alone significantly reduced SOD activity by 16%. There was a significant difference between the three treatments (control, mannitol, NaCl) and the mannitol + NaCl treatment. Thus, we found that SOD activity in roots of mannitol + NaCl-treated plants was significantly higher than in the other treatments (Fig. 2a).

To determine whether the increase in the SOD activity was due to induction of new isoforms or to an increase in its constitutive activity, we subjected tissue extracts to native PAGE. The zymogram of SOD showed 11 different bands (Fig. 3). Seven of these isozymes were identified as Mn-SOD by their insensitivity to KCN and H_2O_2, whereas four of them were inhibited by H_2O_2 and KCN, suggesting that they represented Cu/Zn-SOD activity (data not shown). The SOD isozymes were identified according to their increasing mobility on gels such as Mn-SOD1, Mn-SOD2, Mn-SOD3, Mn-SOD4, Mn-SOD5, Mn-SOD6, Mn-SOD7, Cu/Zn-SOD1, Cu/Zn-SOD2, Cu/Zn-SOD3, and Cu/Zn-SOD4. Nine SOD activity bands (all isozymes except for Mn-SOD2 and Cu/Zn-SOD1) were identified in all treatments. However, mannitol treatment under optimal growth conditions caused the appearance of two distinct bands of Mn-SOD2 and Cu/Zn-SOD1. The intensities of the Mn-SOD3, Mn-SOD4, Mn-SOD5, Mn-SOD7, and Cu/Zn-SOD2 isoforms increased only in plants treated with mannitol under optimal growth conditions. Interestingly, a significant enhancement also occurred in the intensities of Mn-SOD3, Cu/Zn-SOD3, and Cu/Zn-SOD4 in plants treated with mannitol under salt stress compared to the intensities in salt-treated plants alone. Fe-SOD activity was not observed in root extracts. From these results it may be concluded that mannitol treatment significantly enhanced SOD activity by activation of already synthesized enzyme isoforms such as Mn-SOD3, Cu/Zn-SOD3, and Cu/Zn-SOD4 and by inducing the synthesis of new isoforms such as Mn-SOD2 and Cu/Zn-SOD1.

Salt stress increased POX activity (2.5-fold) ($P < 0.001$). Similarly, POX activity was significantly increased (2-fold) by pretreatment with mannitol under salt stress (Fig. 2b). However, mannitol treatment under optimal growth conditions did not cause any change in POX activity. To analyze the changes in POX isozymes in the roots of mannitol-treated Kızıltan-91 under salt stress, they were subjected to native PAGE. Five POX activity bands were identified in all treatments (Fig. 4). Mannitol treatment did not have any effect on POX3, POX4, and POX5 isozymes, although it decreased the intensities of POX1 and POX2 isozymes under optimal growth conditions compared to those in the control group. Interestingly, mannitol treatment under salt stress increased the intensities of all POX isozymes. From these results it seems that mannitol treatment under salt stress enhanced POX activity by increasing the activation of already synthesized POX enzyme isoforms.

Although CAT activity decreased (20%), APX activity increased (16%) under salt stress, in comparison to the control group (Fig. 2c, d). On the other hand, mannitol pretreatment under salt stress increased the activities of CAT and APX by 70% and 55%, respectively. Therefore, the inhibition of CAT activity caused by salinity was prevented by pretreatment with mannitol.

Exogenous application of mannitol did not affect GR activity under optimal growth conditions (Fig. 2e). On the other hand, salt stress inhibited GR activity of Kızıltan-91 by 32%. However, mannitol treatment enhanced GR activity under salt stress. Therefore, mannitol can alleviate the decrease in GR activity in the roots of Kızıltan-91 caused by salinity.

Discussion

Recent work on organisms transformed with genes responsible for the biosynthesis of mannitol, such as those coding for mannose-6-phosphate reductase (M6PR) in celery and mannitol-1-phosphate dehydrogenase (mtID) in

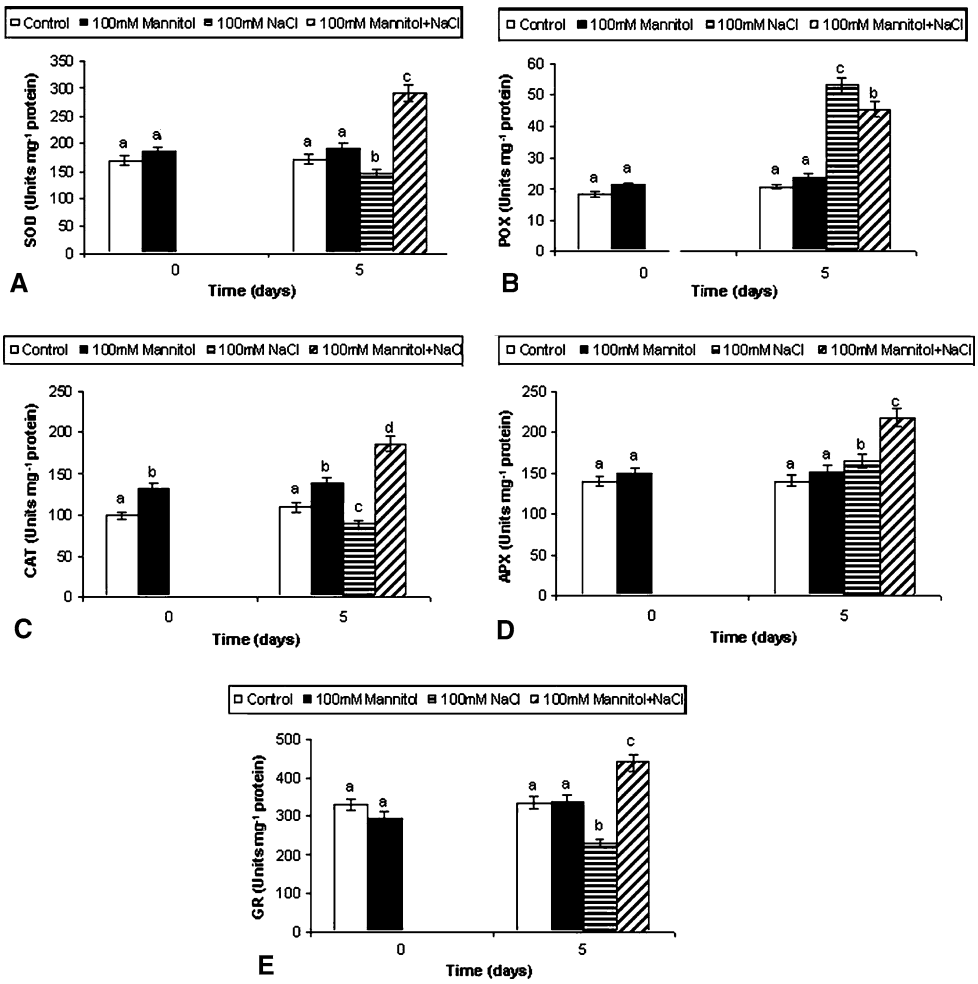

Fig. 2 Effect of exogenous mannitol on the activities of SOD (**a**), POX (**b**), CAT (**c**), APX (**d**), and GR (**e**) (units mg^{-1} protein) in roots of Kızıltan-91 before and after exposure to salt. Values are mean ± SE of six replicates ($n = 6$). Bars with different letters are significantly different at $P < 0.05$

Escherichia coli, strongly suggests that mannitol has specific roles such as a ROS scavenger, stabilizer of proteins and membrane structure, low-molecular-weight chaperone, and protector of photosynthesis under salt and drought stresses (Bohnert and Jensen 1996; Stoop and others 1996; Shen and others 1997; Sickler and others 2007). In addition to the data mentioned above, in the present study we also observed that exogenous application of mannitol enhances activities of antioxidant enzymes in roots of a salt-sensitive wheat cultivar (Kızıltan-91) under salt stress.

Our results demonstrate that exogenous application of mannitol improved the root length of salt-sensitive Kızıltan-91 under salt stress. These findings are in agreement with earlier studies that used the *mtID* genes in tobacco (Tarczynski and others 1992, 1993; Karakas and others 1997; Shen and others 1997), *Arabidopsis* (Thomas and others 1995), and wheat (Abebe and others 2003).

It is known that mannitol added to media can be taken up and used as a source of carbon and energy by plants, but at the same time it changes the water potential of the medium (Lipavska and Vreugdenhil 1996; Conde and others 2007). Therefore, it affects dry matter accumulation of plants. In this study we found that exogenous application of mannitol had no effect on root dry weight under optimal growth conditions. However, mannitol treatment prevented the reduction that occurred in the root dry weight of salt-sensitive wheat under salt stress. In accordance with our results, Karakas and others (1997) found that the dry weight of transgenic tobacco (+*mtID*) plants was increased by mannitol treatment under salt stress compared to that of salt-treated untransformed tobacco.

It is also known that wheat (*Triticum aestivum* L.) cannot synthesize mannitol. However, Lipavska and Vreugdenhil (1996) showed that mannitol is taken up from the medium by in vitro grown plants and that it is transported to shoots rather quickly. Similarly, according to the present study, Kızıltan-91 can incorporate exogenously applied mannitol to significant levels without any signs of toxicity (Table 2). Moreover, higher mannitol content in roots was found in mannitol + NaCl-treated plants. These results are in good agreement with results using tobacco (Stoop and others 1996), wheat (Abebe and others 2003), and *Populus tomentosa* (Hu and others 2005), which all found increased levels of mannitol in these plants under

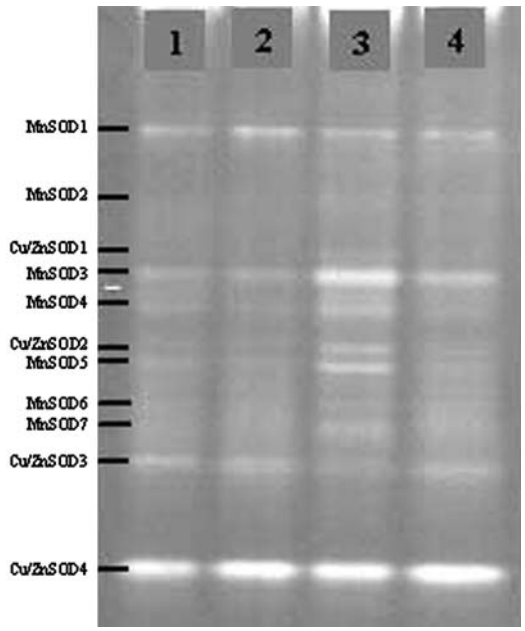

Fig. 3 Effect of exogenous mannitol on activities of SOD isozymes in root tissues of wheat seedlings after salt treatments. Lanes 1, 2, 3, and 4 indicate SOD isozyme activities in control, 100 mM NaCl, 100 mM mannitol, and 100 mM mannitol + NaCl treatment groups, respectively

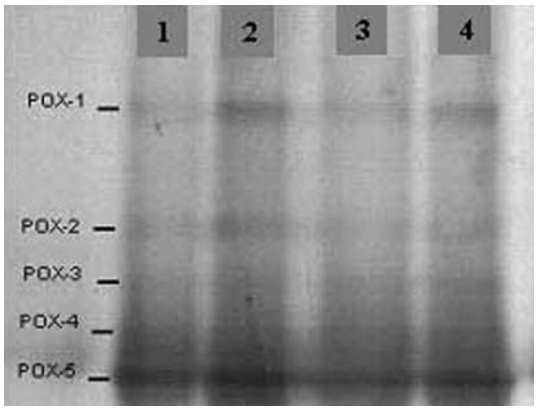

Fig. 4 Effect of exogenous mannitol on activities of POX isozymes in root tissues of wheat seedlings after salt treatments. Lanes 1, 2, 3, and 4 indicate POX isozyme activities in control, 100 mM NaCl, 100 mM mannitol, and 100 mM mannitol + NaCl treatment groups, respectively

salt stress. Moreover, in recent studies of transgenic plants, Tarczynski and others (1992) and Abebe and others (2003) found that tobacco and a wheat cultivar (Bobwhite) accumulated mannitol in leaves and roots under salt stress and exhibited a highly protective mechanism against oxidative damage caused by salt stress.

It is well established that peroxidation of lipid membranes of higher plants reflects free radical-induced oxidative damage at the cellular level under salt stress (Hernandez and others 2001; Jain and others 2001; Demiral and Türkan 2005; Yin and others 2008). In the present study, a significant increase was observed in the lipid peroxidation level of Kızıltan-91 roots under salt stress. Dhindsa and others (1981) have reported that an increase in lipid peroxidation under salinity is a reflection of cell membrane injury. This is reported to be due to accumulated free radicals and inadequate activities of antioxidant enzymes (Sairam and others 1998). Nevertheless, the decrease of lipid peroxidation in roots of mannitol + NaCl-treated wheat plants that was observed in this study suggests that exogenous application of mannitol provides a protective mechanism by scavenging reactive oxygen species and increasing the activities of SOD, POX, CAT, APX, and GR against salt-dependent oxidative damage. Consistent with this idea, Shen and others (1997) and Sickler and others (2007) have reported that mannitol functions as a scavenger of reactive oxygen species thereby preventing peroxidation of lipids resulting in cell damage.

SOD is an enzyme that catalyzes the conversion of the superoxide radical to molecular oxygen and H_2O_2 (Scandalios 1993). In the present study, the activity of SOD showed a significant decrease under salt stress. This is consistent with previous work by Dionisio-Sese and Tobita (1998) who reported that a decrease in SOD activity under salt stress led to an accumulation of superoxide radicals that could cause membrane damage. However, exogenous application of mannitol significantly enhanced SOD activity in plants under salt stress. We identified 11 SOD isozymes in wheat roots consisting of Mn-SOD and Cu/Zn-SOD isozymes. Our results indicate that SOD isozymes in the roots were affected differently by pretreatment with mannitol under salt stress. In particular, the intensities of Cu/Zn-SOD3, Cu/Zn-SOD4, and Mn-SOD1 increased more in the roots of mannitol-treated plants (mannitol and mannitol + NaCl) than in plants subjected to other treatments. Interestingly, additional isozymes (Mn-SOD2 and Cu/Zn-SOD1) were induced only in mannitol-treated plants.

POXs are involved not only in scavenging of H_2O_2 but also in growth, developmental, lignification, and suberization processes (Dionisio-Sese and Tobita 1998; Jbir and others 2001). Salt-tolerant plants are often found to stimulate POX enzymes, which are active in the elimination of hydrogen peroxide (Bor and others 2003; Meloni and others 2003). In the present study, an increase in POX activity was observed consistently in the roots of plants subjected to salt stress whether or not they had been pretreated with mannitol. These results are also consistent with the results of Mandhania and others (2006) who found an increase in the POX activity of two wheat cultivars (KRL-19 and WH-542) under salt stress. The utilization of multiple isoforms of an enzyme is one of the primary control mechanisms of cellular metabolism in plants. In the present study, five specific isozymes were determined in POX in the roots of Kızıltan-

91. Exogenous application of mannitol induced all POX isozymes under salt stress. In the present study, a significant increase in POX activity in plants treated with mannitol under salt stress was highly correlated with the temporal regulation of the specific isoenzymes.

CAT is one of the main H_2O_2-scavenging enzymes that dismutate H_2O_2 into water and O_2, and it is a constitutive component of peroxisomes (Corpas and others 1999). In this study, an enhancement of CAT was observed in plants under salt stress when the plants were subjected to mannitol pretreatment. Similar results were also obtained for the APX activity of Kızıltan-91. Therefore, it may be suggested that both CAT and APX are probably equally important in the detoxification of H_2O_2 in Kızıltan-91. Mannitol caused enhancements in the H_2O_2 scavenging activity of CAT and APX in the roots of salt-sensitive Kızıltan-91**.** Because membrane injury under salt stress is related to an increased production of ROS, enhancement of APX and CAT activities in the roots of mannitol + NaCl-treated plants might have decreased the level of ROS and resulted in a reduced level of lipid peroxidation.

Glutathione reductase (GR) is the last enzyme of the ascorbate-glutathione cycle. This enzyme catalyzes NADPH-dependent reduction of oxidized glutathione. GR is important in protecting many plants from oxidative stress (Guy and Carter 1984; Foyer and others 1991). In our study, salt stress was observed to reduce GR activity in roots of Kızıltan-91. Similar results were also reported for the roots of rice (Demiral and Türkan 2005) and *Centaurea ragusina* (Radić and others 2006). As mannitol increased both APX and GR activities under salt stress, it may be considered that the effects of oxidative stress may be diminished by the activity of the ASC-GSH cycle.

In conclusion, mannitol pretreatment under salt stress induced increases in the activities of SOD, CAT, and APX in the roots of salt-sensitive wheat plants. Concordantly, mannitol pretreatment protected the roots against lipid peroxidation under salt stress. These results suggest that exogenous application of mannitol could protect wheat plants from the harmful effects of salt-induced oxidative stress by enhancing the activity of antioxidant enzymes.

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References

Abebe T, Guenzi A, Martin B, Cushman JC (2003) Tolerance of mannitol-accumulating transgenic wheat to water stress and salinity. Plant Physiol 131:1748–1755

Bergmeyer N (1970) Methoden der enzymatischen, Analyse, vol 1. Akademie Verlag, Berlin, pp 636–647

Beuchamp C, Fridovich I (1971) Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Ann Biochem 44:276–287

Beuchamp C, Fridovich I (1973) Isozymes of superoxide dismutase from wheat germ. Biochim Biophys Acta 317:50–64

Beutler HO (1988) Xylitol. In: Bergmeyer HU (ed), Methods of enzymatic analysis, 3rd edn., vol VI. VCH Publishers Ltd., Cambridge, p 487

Bohnert HJ, Jensen RG (1996) Strategies for engineering water-stress tolerance in plants. Trends Biotechnol 14:89–97

Bohnert HJ, Nelson DE, Jensen RG (1995) Adaptations to environmental stresses. Plant Cell 7:1099–1111

Bor M, Ozdemir F, Türkan I (2003) The effect of salt stress on lipid peroxidation and antioxidants in leaves of sugar beet *Beta vulgaris* L. and wild beet *Beta maritima* L. Plant Sci 164:77–84

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Conde C, Silva P, Agasse A, Lemoine R, Delrot S, Tavares R, Geros H (2007) Utilization and transport of mannitol in *Olea europea* and implications for salt stress tolerance. Plant Cell Physiol 48:42–53

Corpas FJ, Palma JM, Sandalio LM, Lopez-Huertas E, Romero-Puertas MC, Barroso JB, Del Rio LA (1999) Purification of catalase from pea leaf peroxisomes: identification of five different isoforms. Free Radical Res 31:235–241

Demiral T, Türkan I (2005) Comparative lipid peroxidation, antioxidant defense systems and proline content in roots of two rice cultivars differing in salt tolerance. Environ Exp Bot 53:247–257

Dhindsa RS, Plumb-Dhindsa P, Thorne PA (1981) Leaf senescence correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. J Exp Bot 32:93–101

Dionisio-Sese ML, Tobita S (1998) Antioxidant responses of rice seedlings to salinity stress. Plant Sci 135:1–9

Everard JD, Franceshi VR, Loescher WH (1993) Mannose-6-phosphate reductase, a key enzyme in photoassimilate partitioning, is abundant and located in the cytosol of photosynthetically active cells of celery (*Apium graveolens* L.) source leaves. Plant Physiol 102:345–356

Foyer CH, Halliwell B (1976) The presence of glutathione and glutathione reductase in chloroplast: a proposed role in ascorbic acid metabolism. Planta 133:21–25

Foyer CH, Lelandais M, Galap C, Kunert KJ (1991) Effect of elevated cytosolic glutathione reductase activity on the cellular glutathione pool and photosynthesis in leaves under normal and stress conditions. Plant Physiol 97:863–872

Greenway H, Munns R (1980) Mechanisms of salt tolerance in non-halophytes. Annu Rev Plant Physiol 31:149–190

Guy CL, Carter JV (1984) Characteristic of partially purified glutathione reductase from cold-hardened and non-hardened spinach leaf tissue. Cyrobiology 21:454–464

Hernandez JA, Jiménez A, Mullineaux P, Sevilla F (2000) Tolerance of pea (*Pisum sativum* L.) to long-term salt stress is associated with induction of antioxidant defences. Plant Cell Environ 23:853–862

Hernandez JA, Ferrer MA, Jimenez A, Barcelo AR, Sevilla F (2001) Antioxidant systems and $O_2^{\bullet -}/H_2O_2$ production in the apoplast of pea leaves Its relation with salt-induced necrotic lesions in minor veins. Plant Physiol 127:817–831

Herzog V, Fahimi H (1973) Determination of the activity of peroxidase. Anal Biochem 55:554–562

Hu L, Lu H, Liu QL, Chen XM, Jiang XN (2005) Overexpression of *mtID* gene in transgenic *Populus tomentosa* improves salt tolerance through accumulation of mannitol. Tree Physiol 25:1273–1281

Jain M, Mathur G, Konl S, Sarin NB (2001) Ameliorative effects of proline on salt stress lipid peroxidation in cell lines of groundnut (*Arachis hypogea* L.). Plant Cell Rep 20:463–468

Jbir N, Chaibi W, Amar S, Jemmali A, Ayadi A (2001) Root growth and lignification of two wheat species differing in their sensitivity to NaCl in response to salt stress. CR Acad Sci Paris 324:863–868

Karakas B, Ozıas-Akıns P, Stushnoff C, Suefferheld M, Rieger M (1997) Salinity and drought tolerance of mannitol-accumulating transgenic tobacco. Plant Cell Environ 20:609–616

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Lipavska H, Vreugdenhil D (1996) Uptake of mannitol from the media by in vitro grown plants. Plant Cell Tiss Org 45:103–107

Lunn PG, Nothrop CA, Nothrop AJ (1989) Automated enzymatic assays for the determination of intestinal permeability probes in urine. 2. Mannitol. Clin Chim Acta 183:163–170

Madhava Rao KV, Sresty TVS (2000) Antioxidative parameters in the seedlings of pigeon pea (*Cajanus cajan* L. Millspaugh) in response to Zn and Ni stresses. Plant Sci 157:113–128

Mandhania S, Madan S, Sawhney V (2006) Antioxidant defense mechanism under salt stress in wheat seedlings. Biol Plant 2:227–231

Marschner H (1995) Adaptations of plants to adverse chemical soil conditions. In: Mineral nutrition of higher plants. Academic Press, New York, pp 596–680

Meloni DA, Oliva MA, Martinez CA, Canbraia J (2003) Photosynthesis and activity of superoxide dismutase, peroxidase and glutathione reductase in cotton under salt stress. Environ Exp Bot 49:69–76

Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci 7:405–410

Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol 22:867–880

Noiraud N, Maurousset L, Lemoine R (2001) Identification of a mannitol transporter, AgMaT1, in celery phloem. Plant Cell 13:695–705

O'Neill SD (1983) Osmotic adjustment and the development of freezing resistance in *F. virginiana*. Plant Physiol 72:938–944

Radić S, Radić-Stojković M, Pevalek-Kozlina B (2006) Influence of NaCl and mannitol on peroxidase activity and lipid peroxidation in *Centaurea ragusina* L. roots and shoots. J Plant Physiol 163:1284–1292

Sairam RK, Deshmukh PS, Saxena DC (1998) Role of antioxidant systems in wheat genotypes tolerance to water stress. Biol Plant 41:387–394

Scandalios JG (1993) Oxygen stress and superoxide dismutase. Plant Physiol 101:7–12

Seevers FM, Daly JM, Catedral FF (1971) The role of peroxidase isozymes in resistance to wheat stem rust. Plant Physiol 48:353–360

Sekmen AH, Türkan I, Takio S (2007) Differential responses of antioxidative enzymes and lipid peroxidation to salt stress in salt-tolerant *Plantago maritima* L. and salt-sensitive *Plantago media* L. Physiol Plant 131:399–411

Shen B, Jensen RG, Bohnert HJ (1997) Mannitol protects against oxidation by hydroxyl radicals. Plant Physiol 115:527–532

Sickler CM, Edwards GE, Kiirats O, Gao Z, Loescher W (2007) Response of mannitol-producing *Arabidopsis thaliana* to abiotic stress. Funct Plant Biol 34:382–391

Smirnoff N, Cumbes QJ (1989) Hydroxyl radical scavenging of compatible solutes. Phytochemistry 28:1057–1060

Stoop JMH, Williamson JD, Pharr DM (1996) Mannitol metabolism in plants: a method for coping with stress. Trends Plant Sci 1:139–144

Tarczynski MC, Jensen RG, Bohnert HJ (1992) Expression of a bacterial *mtID* gene in transgenic tobacco leads to production and accumulation of mannitol. Proc Natl Acad Sci USA 89:2600–2604

Tarczynski MC, Jensen RG, Bohnert HJ (1993) Stress protection of transgenic tobacco by production of the osmolyte mannitol. Science 259:508–510

Tholakalabavi A, Zwiazek JJ, Thorpe TA (1994) Effect of mannitol and glucose-induced osmotic stress on growth, water relations and solute composition of cell suspension culture of poplar (*Poplus deltoides* var. *occidentalis*) in relation to anthocyanin accumulation. In Vitro Cell Dev Biol Plant 30:164–170

Thomas JC, Seaphi M, Arendall B, Bohnert HJ (1995) Enhancement of seed germination in high salinity by engineering mannitol expression in *Arabidopsis thaliana*. Plant Cell Environ 18:801–806

Trip P, Krotkov G, Nelson CD (1964) Metabolism of mannitol in higher plants. Am J Bot 51:828–835

Vitória AP, Lea PJ, Azevedo RA (2001) Antioxidant enzymes responses to cadmium in radish tissues. Phytochemistry 57:701–710

Williamson JD, Jennings DB, Guo W-E, Pharr DM, Ehrenshaft M (2002) Sugar alcohols, salt stress and fungal resistance: polyols-multifunctional plant protection? J Am Soc Hort Sci 127:467–473

Yin H, Chen Q, Yi M (2008) Effects of short-term heat stress on oxidative damage and responses of antioxidant system in *Lilium longiflorum*. Plant Growth Regul 54:45–54