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Exogenously applied ascorbic acid alleviates salt-induced oxidative stress in wheat

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Abstract

Although ascorbic acid (AsA) is one of the most important and abundantly occurring water soluble antioxidants in plants, relatively little is known about its role in counteracting the adverse effects of salt stress on plant growth. To address this issue that whether exogenous application of ascorbic acid (AsA) through rooting medium could alleviate the adverse effects of salt stress on wheat plants, a hydroponic experiment was conducted under glasshouse conditions using two wheat cultivars, S-24 (salt tolerant) and MH-97 (moderately salt sensitive). Plants of both cultivars were subjected to 0 or 150 mM NaCl solution supplemented with 0, 50, or 150 mg L^{-1} AsA for 58 days. Imposition of salt stress reduced the growth of both wheat cultivars by causing reduction in photosynthesis, and endogenous AsA level, and enhancing accumulation of Na^+ and Cl^- coupled with a decrease in K^+ and Ca^{2+} in the leaves and roots of both cultivars thereby decreasing tissue K^+/Na^+ ratio. However, root applied AsA counteracted the adverse effects of salt stress on the growth of cv. S-24 only, particularly at 100 mg L^{-1} AsA level. AsA-induced enhancement in growth of salt-stressed plants of S-24 was associated with enhanced endogenous AsA level and CAT activity, and higher photosynthetic capacity, and accumulation of K^+ and Ca^{2+} in the leaves. Although root applied AsA did not improve the growth of salt-stressed plants of MH-97, it enhanced endogenous level of AsA, CAT activity, photosynthetic capacity, and leaf K^+ and Ca^{2+}. These findings led us to conclude that root applied AsA counteracts the adverse effects of salt stress on growth of wheat by improving photosynthetic capacity of wheat plants against salt-induced oxidative stress and maintaining ion homeostasis, however, these effects were cultivar specific.
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Keywords: Antioxidants; Catalase; Ion homeostasis; K^+/Na^+ ratio; Photosynthesis; Salt tolerance

1. Introduction

Salt stress causes a number of changes in plant metabolism. Of them, ion toxicity, osmotic stress and production of reactive oxygen species (ROS) are most prominent (Mittler, 2002). ROS are generally produced in mitochondria and chloroplasts. In chloroplasts, ROS are generated by direct transfer of excitation energy from chlorophyll to produce singlet oxygen, or by univalent oxygen reduction of photosystem I in the Mehler reaction (Asada, 1999). ROS are highly reactive and in the absence of any protective mechanism they can seriously cripple normal metabolism through oxidative damage to lipids, proteins and nucleic acids. For example, H_2O_2 can down-regulate CO_2 assimilation by inhibiting several Calvin cycle enzymes (Asada, 1999). ROS are also known to serve as signaling intermediates in guard cells to promote stomatal closure (Foyer and Noctor, 2003), cause damage to cell membranes or even provoke apoptosis (Loreto et al., 2001). The generation of ROS is limited or scavenged by an antioxidant system including antioxidant compounds (ascorbate, salicylate, glutathione, tochopherols, etc.) and antioxidant enzymes like superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT) (Foyer and Noctor, 2003). Salt tolerant plants, in addition to regulating the ion and water homeostasis should also have a better antioxidant system for the effective removal of ROS (Mittler, 2002). In view of a number of studies, salt tolerance is often correlated well with a more efficient oxidative system (Gossett et al., 1994, 1996; Noctor and Foyer, 1998; Mittova et al., 2002; Bor et al., 2003).

Ascorbic acid (AsA) is one of the most important antioxidants abundantly occurring in plants (Smirnoff, 2000). Generally, its concentration is higher in leaves than that in other plant parts and it is 5–10 times higher than that of glutathione (GSH) (Smirnoff, 2005). The ability of ascorbate to lose or donate

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electrons to produce MDHA is the basis of its biologically useful antioxidant capacity (Buettner and Schafer, 2004). The role of AsA as an antioxidant has been shown by Müller-Moulé et al. (2003, 2004) who demonstrated that AsA deficient mutants of *Arabidopsis* (*vtc* mutants) were more sensitive to ozone, sulfur dioxide, or UV-B light (Veljovic-Jovanovic et al., 2001). Similarly, *vtc2* mutants of *Arabidopsis* experienced severe high light-induced oxidative stress along with increased lipid peroxidation and photo-inhibition when *vtc2* mutants were transferred from low to high light (Müller-Moulé et al., 2003, 2004). Thus, high endogenous AsA in plants is necessary to counteract oxidative stress in addition to regulating other processes of plant metabolism. Endogenous AsA can be increased by exogenous application of AsA through the rooting medium (Chen and Gallie, 2004), as a foliar spray or as seed priming.

Despite its role in scavenging ROS, AsA is also involved in regulating photosynthetic capacity by controlling stomatal movement (Chen and Gallie, 2004). Ascorbate is also an important co-factor of some enzymes or protein complexes that are involved in the regulation of photosynthesis (Davey et al., 2000). However, detailed information on how AsA can cause changes in photosynthesis remains to be elucidated. In view of the above-mentioned reports, an experiment was conducted to assess whether exogenously applied AsA through rooting medium could alter antioxidant capacity, that may or may not alter ion relations, and photosynthetic capacity of wheat plants.

2. Materials and methods

A hydroponic experiment was conducted during the winter of 2004–2005 in a net-house at the Botanic Gardens of the University of Agriculture, Faisalabad, Pakistan (latitude 31°30′N, longitude 73°10′E and altitude 213 m), with 10/14 light/dark period at 800–1100 μmol m^{-2} s^{-1} PPFD, a day/night average temperature cycle of 26/15 °C and 65 ± 5% relative humidity. Seeds of a salt tolerant (S-24) and a moderately salt sensitive cultivar (MH-97) of spring wheat were obtained from the Department of Botany, University of Agriculture, Faisalabad, Pakistan and Ayub Agricultural Research Institute, Faisalabad, Pakistan, respectively. Seven hundred seeds of each cultivar were surface sterilized with 5% sodium hypochlorite for 10 min and then thoroughly rinsed with distilled water before further experimentation. Surface sterilized seeds of each genotype were sown in plastic pots filled with sand irrigated with full strength Hoagland's nutrient solution containing 0 or 150 mM NaCl. The seeds were allowed to emerge for 8 days. Twenty 8-day-old wheat seedlings (five plants per replicate) of uniform size of each genotype were then transferred to plastic tubs (45 cm × 66 cm × 23 cm) containing 20 L of different concentration of ascorbic acid (0, 50, or 100 mg L^{-1}) in full strength Hoagland's nutrient solution with or without 150 mM NaCl. During the first week of acclimatization, if any plant wilted it was replaced with a healthy one. The treatment solutions in all tubs were continuously aerated. Following physiological attributes were measured 58 days after sowing.

2.1. Gas exchange parameters

An open system LCA-4 ADC portable infrared gas analyzer (Analytical Development Company, Hoddesdon, England) was used for the measurements of gas exchange parameters such as net CO_2 assimilation rate (A), transpiration (E), sub-stomatal conductance (C_i) and stomatal conductance (g_s). All measurements were made on a fully expanded third leaf from top of each plant. Measurements were performed from 9.00 to 13.00 h with the following specifications/adjustments: molar flow of air per unit leaf area 406.3 μmol m^{-2} s^{-1}, atmospheric pressure 98.6 kPa, water vapor pressure into the chamber ranged from 6.0 to 8.9 mbar, PAR at the leaf surface was maximum up to 1889 μmol m^{-2} s^{-1}, temperature of the leaf ranged from 28.4 to 32.4 °C, ambient temperature ranged from 22.4 to 27.9 °C, and ambient CO_2 concentration was 352 μmol mol^{-1}.

2.2. Extraction of antioxidant enzymes

Fresh leaves (0.5 g of third leaves) of both wheat cultivars were ground in 8 mL of 50 mM cold phosphate buffer (pH 7.8) and centrifuged at 15,000 × g for 20 min at 4 °C. The supernatant was used for the determination of the activities of antioxidant enzymes.

2.3. Superoxide dismutase (SOD)

The activity of SOD was assayed following the method of Giannopolitis and Ries (1977) which measures its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT). The reaction solution (3 mL) contained 50 μM NBT, 1.3 μM riboflavin, 13 mM methionine, 75 nM EDTA, 50 mM phosphate buffer (pH 7.8), and 20–50 μL enzyme extract. The test tubes containing the reaction solutions were irradiated under a light (15-W fluorescent lamps) at 78 μmol m^{-2} s^{-1} for 15 min. The absorbance of the irradiated solution at 560 nm was determined with a spectrophotometer (Hitachi U-2100, Tokyo, Japan). One unit of SOD activity was defined as the amount of enzyme which caused 50% inhibition of photochemical reduction of NBT.

2.4. Catalase (CAT) and peroxidase (POD)

Activities of CAT and peroxidase (POD) were appraised following the method of Chance and Maehly (1955) with some modification. The CAT reaction solution (3 mL) contained 50 mM phosphate buffer (pH 7.0), 5.9 mM H_2O_2, and 0.1 mL enzyme extract. The reaction was initiated by adding the enzyme extract. Changes in absorbance of the reaction solution at 240 nm were read every 20 s. One unit CAT activity was defined as an absorbance change of 0.01 units/min. The POD reaction solution (3 mL) contained 50 mM phosphate buffer (pH 5.0), 20 mM guaiacol, 40 mM H_2O_2, and 0.1 mL enzyme extract. Changes in absorbance of the reaction solution at 470 nm were determined every 20 s. One unit POD activity was defined as an absorbance change of 0.01 units/min.

The activity of each enzyme was expressed on protein basis. Protein concentration of the crude extract was measured by the method of Bradford (1976).

2.5. Ascorbic acid

Ascorbic acid was determined as described by Mukherjee and Choudhuri (1983). Leaf material (0.25 g of the third leaf) was extracted with 10 mL of 6% trichloroacetic acid. Four milliliters of the extract were mixed with 2 mL of 2% dinitrophenyl hydrazine (in acidic medium) followed by the addition of one drop of 10% thiourea (in 70% ethanol). The mixture was boiled for 15 min in a water bath and after cooling at room temperature, 5 mL of 80% (v/v) H_2SO_4 were added to the mixture at 0 °C. The absorbance was read at 530 nm. The concentration of ascorbic acid was calculated from a standard curve plotted with known concentrations of ascorbic acid.

Fifty-eight days after sowing two plants from each replicate were harvested. Shoots and roots were weighed for fresh weights and then all samples were oven-dried at 65 °C for 1 week for recording dry weights.

2.6. Determination of mineral elements in plant tissues

Na^+, K^+ and Ca^{2+} in the leaves and roots were determined by the methods described by Allen et al. (1986). Ground dry plant samples (100 mg each) were digested in 2 mL of sulfuric-peroxide digestion mixture until a clear and almost colorless solution was obtained. After digestion, the volume of each sample was made to 100 mL with distilled de-ionized water. Ions, i.e. Na^+, K^+ and Ca^{2+} were determined with a flame photometer (Jenway PFP7). For Cl^- analysis, 100 mg of ground shoot and root samples were extracted in 10 mL of distilled water at 80 °C for 4 h. Cl^- concentration was determined with a chloride analyzer (Corning, 925).

2.7. Statistical analysis of data

The data for each variable was subjected to analysis of variance using the COSTAT computer package (Cohort Software, Berkeley, CA). The mean values were compared with the least significance difference test following Snedecor and Cochran (1980).

3. Results

Salt stress reduced the shoot fresh and dry weights of both wheat cultivars. Although exogenous application of AsA improved the shoot fresh and dry weights of both cultivars under non-saline conditions, under saline conditions it only improved shoot fresh and dry weight of cv. S-24, particularly at 100 mg L^{-1} AsA level (Fig. 1).

Salt stress significantly reduced leaf ascorbic acid contents of both cultivars. Exogenous application of ascorbic acid through the rooting medium increased ascorbic acid contents in the stressed and non-stressed plants of both cultivars (Fig. 2).

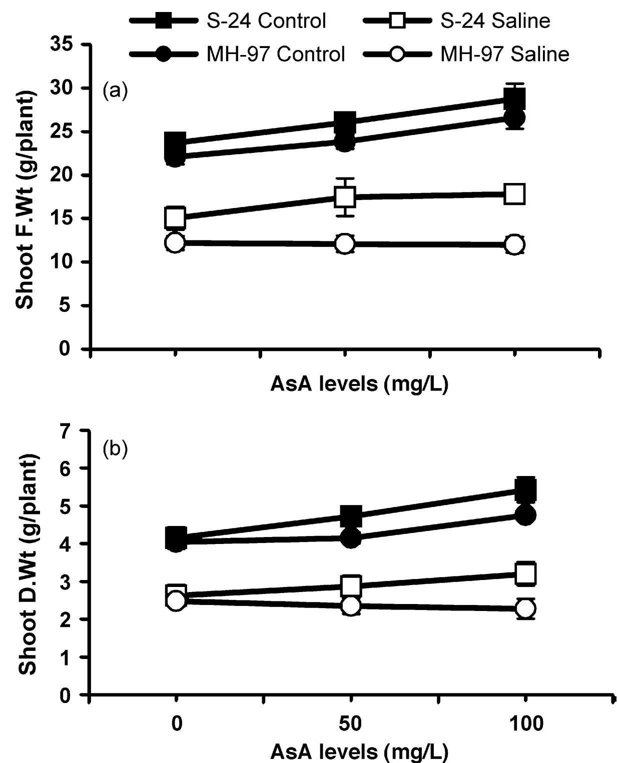

Fig. 1. Shoot fresh and dry weight of salt-stressed and non-stressed plants of two spring wheat cultivars when different concentrations of ascorbic acid were applied through the rooting medium.

Salt-stressed plants of both cultivars showed higher activities of superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) than those in non-stressed plants (Fig. 2). Ascorbic acid applied through the rooting medium significantly increased the CAT activity in the salt-stressed plants of both wheat cultivars, particularly at 100 mg L^{-1} AsA. However, POD activity of salinized plants of both cultivars was not much affected due to AsA applied through the rooting medium. In contrast, 50 mg L^{-1} AsA caused reduction in the SOD activity of the salt-stressed plants of both cultivars.

Saline growth medium caused a significant reduction in all gas exchange attributes except sub-stomatal CO_2 (C_i), which remained almost unaffected (Fig. 3). However, salt-stressed plants of cv. S-24 exhibited higher A than that of MH-97. Exogenous application of AsA through the rooting medium increased the CO_2 assimilation rate in both the stressed and non-stressed plants of both cultivars. In addition, AsA-induced improvement in photosynthetic rate of non-stressed plants of both cultivars was consistent with increasing levels of AsA in the rooting medium (Fig. 3). Transpiration rate (E) of cv. S-24 increased due to root applied AsA under non-saline or saline conditions, whereas in MH-97 it increased in non-stressed plants only. Although root applied AsA caused an increase in stomatal conductance (g_s) of both cultivars under saline conditions, changes in g_s along with sub-stomatal CO_2 (C_i) were only observed in the salt-stressed plants of cv. S-24. Furthermore, water use efficiency (calculated as A/E WUE) of non-stressed plants of both cultivars was increased due to 100 mg L^{-1} AsA applied through the rooting medium, but this was not true for the stressed plants.

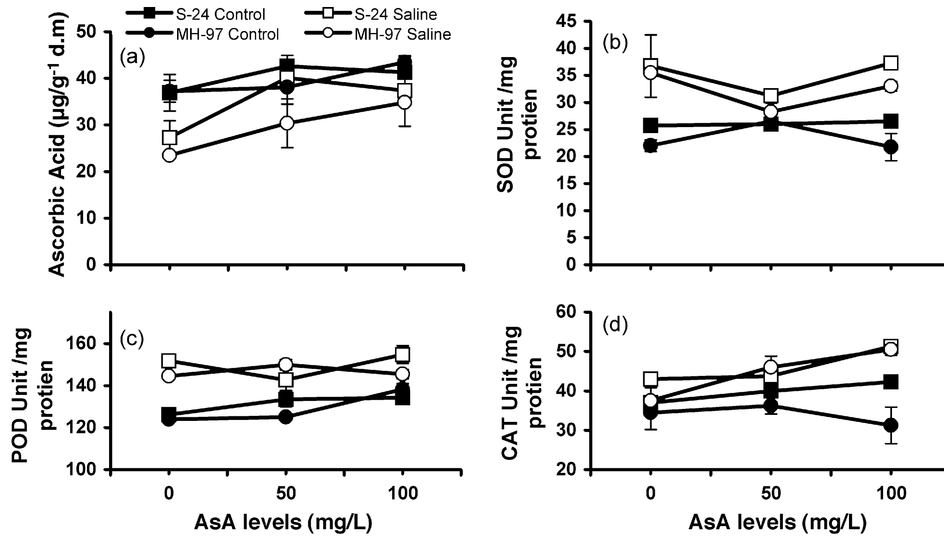

Fig. 2. Ascorbic acid contents, and activities of SOD, POD and CAT at the booting stage of salt-stressed and non-stressed plants of two spring wheat cultivars when different concentrations of ascorbic acid were applied through the rooting medium.

Accumulation of Na^+ and Cl^- in the leaves and roots of both wheat cultivars was significantly increased under saline conditions (Fig. 4). Exogenous application of 50 mg L^{-1} AsA through the rooting medium enhanced the accumulation of Na^+ and Cl^- in the leaves of salt-stressed plants of cv. S-24 (Fig. 4). However, 100 mg L^{-1} AsA enhanced the accumulation of both Na^+ and Cl^- in the roots of salinized plants of MH-97.

Accumulation of K^+ and Ca^{2+} in the leaves and roots of both cultivars was significantly reduced due to salt stress (Fig. 4). However, application of 100 mg L^{-1} AsA through the rooting medium enhanced the accumulation of K^+ in the leaves and roots of the salt-stressed plants of both cultivars. Similarly, accumulation of Ca^{2+} in the leaves of salinized plants of both cultivars was also enhanced due to 50 or 100 mg L^{-1} AsA application. In contrast, in the salt-stressed plants of both cultivars, root Ca^{2+} was not significantly affected due to AsA application.

Saline growth medium caused a significant reduction in leaf and root K^+/Na^+ ratios in both wheat cultivars. Exogenously applied 50 mg L^{-1} ascorbic acid through the rooting medium enhanced the leaf K^+/Na^+ ratio in both stressed and non-stressed plants of cv. S-24 (Fig. 4), while it caused reduction in root K^+/Na^+ ratio in the salt-stressed plants of both wheat cultivars.

If we draw the relationship between shoot fresh and dry weight and accumulation of ions in leaves and roots, it is clear that both shoot fresh and dry weight is positively associated with the K^+, Ca^{2+} and K^+/Na^+ ratio in the leaves and roots (r = 0.725***, 0.468***, 0.583***, 0.729***, 0.784***, and 0.750***, respectively), and negatively associated with the accu-

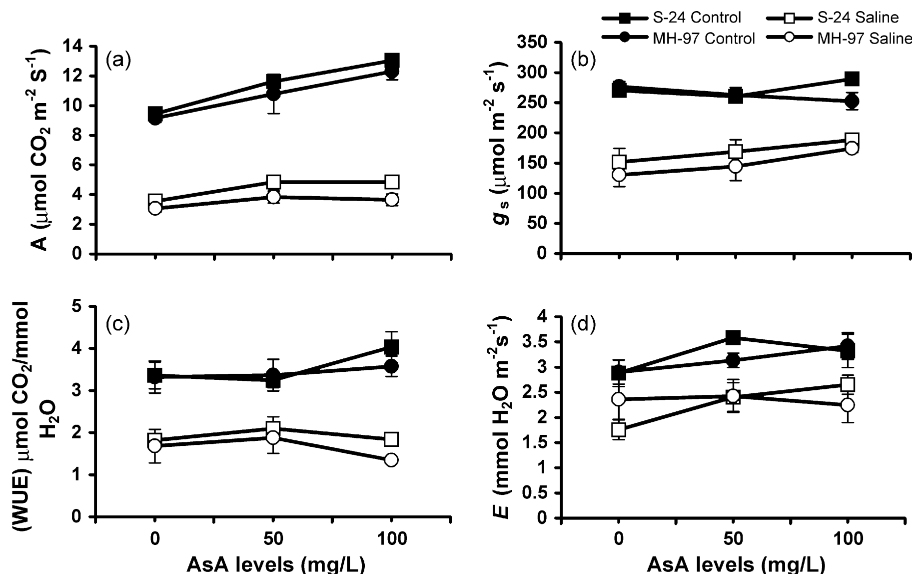

Fig. 3. Gas exchange characteristics of salt-stressed and non-stressed plants of two spring wheat cultivars when different concentrations of ascorbic acid were applied through the rooting medium.

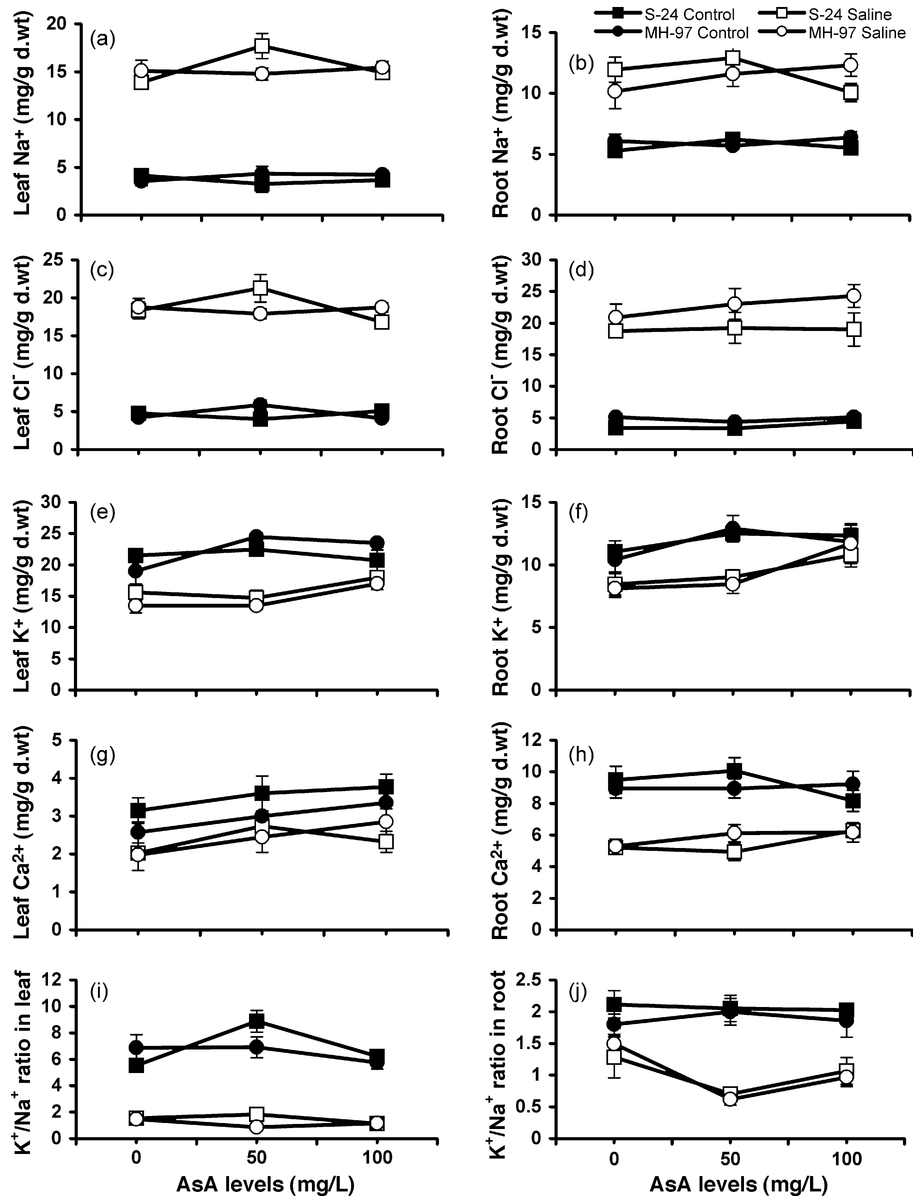

Fig. 4. Na^+, K^+, Ca^{2+}, and Cl^- concentration in the leaves and roots of salt-stressed and non-stressed plants of two spring wheat cultivars when different concentrations of ascorbic acid were applied through the rooting medium.

mulation of Na^+ in the leaves and roots ($r = -0.837^{***}$ and -0.708^{***}, respectively).

4. Discussion

Plants under salt stress use a variety of strategies to counteract the adverse effects of salt stress on their growth. Of these, ion homeostasis, enhanced antioxidant activity, and enhanced photosynthetic capacity are prominent. It is now well evident that salt tolerance in most crop plants is associated with a more efficient antioxidant system (Gossett et al., 1994, 1996; Noctor and Foyer, 1998; Mittova et al., 2002; Bor et al., 2003). An efficient antioxidant system includes enzymic (SOD, APX, and CAT), and non-enzymic antioxidants (ascorbate, tocopherols, salicylic acid, and carotenoids). Ascorbic acid (AsA) is one of the most important antioxidants protecting plants from oxidative stress (Smirnoff, 2005). AsA is also involved in regulating photosynthetic capacity, flowering and senescence (Davey et al., 2000), and in counteracting adverse effects of salt stress in tomato (Shalata and Neumann, 2001) and wheat (Al-Hakimi and Hamada, 2001). In the present study, exogenously applied AsA through the rooting medium have caused enhancement in growth of non-stressed plants of both wheat cultivars. However, in salt-stressed plants growth of only salt tolerant cv. S-24 improved due to exogenously applied AsA through the rooting medium. These findings suggest that ameliorative effect of AsA on growth of wheat plants was cultivar specific. However, AsA-induced increase in growth of wheat plants under non-saline or saline conditions may have been due to increase in cell division and/or cell enlargement, although these phenomena were not examined in the present study. Furthermore, there are

some reports which provide evidence that AsA accelerates cell division and cell enlargement as observed in different plants such as *Pisum* (Cabo et al., 1996), and *Lupinus albus* (Citterio et al., 1994). These findings and the results of the present study suggest that growth promoting effect of AsA under non-saline or saline conditions may have been due to enhanced antioxidant capacity, and increase in cell division and cell enlargement.

In the present study, salt stress caused a reduction in the endogenous levels of ascorbic acid in the leaves of both cultivars (Fig. 2a). This salt-induced reduction in AsA may have been due to its direct destruction by ROS as has earlier been observed in pea under water stress conditions (Iturbe-Ormaetxe et al., 1998). However, exogenous application of AsA through the rooting medium enhanced the endogenous level of AsA in both cultivars. Furthermore, salt tolerant S-24 maintained higher endogenous AsA level than that in the moderately salt sensitive MH-97. Because it is known that AsA can directly scavenge superoxide, hydroxyl radicals, singlet oxygen and H_2O_2 to water via ascorbate peroxidase reaction (Noctor and Foyer, 1998), high level of endogenous ascorbate is essential to maintain the antioxidant system that protects plants from oxidative damage due to different types of stresses (Shigeoka et al., 2002). Now it is well evident that plant cells are strongly redox buffered due to very large quantities of the water soluble antioxidants including ascorbate (10–100 mM) (Noctor and Foyer, 1998; Foyer and Noctor, 2003; 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.

During photosynthesis superoxide radicals are produced due to direct electron transfer to oxygen, from which H_2O_2 is produced (Mittler, 2002). However, intercellular level of H_2O_2 is regulated by a wide range of enzymes. Of them, CAT (Willekens et al., 1995) and PODs are more important (Foyer and Noctor, 2003). In the present study, salt stress increased the activities of SOD, CAT, and POD in both cultivars, which indicates that wheat cultivars up-regulate SOD–CAT–POD antioxidative system so as to scavenge ROS. However, exogenous application of AsA, particularly 100 mg L^{-1} AsA, only enhanced the CAT activities of both cultivars under saline conditions, being considerably higher in S-24 than that in MH-97 due to AsA application. These findings about the behavior of the antioxidant enzymes from the present study suggest that an accumulation of ROS might have occurred in response to salt stress which was reduced by enhanced catalase (CAT) and endogenous AsA level due to the application of AsA. This view is further supported by the arguments that major detoxification of ROS produced during photosynthesis are mediated by CAT and by reductive processes involving the major redox buffers of plant cells such as ascorbate and glutathione (Noctor and Foyer, 1998; Noctor et al., 2002; Foyer and Noctor, 2003). Furthermore, such a mechanism may be important in certain abiotic stresses which decrease stomatal conductance such as drought, salinity, high temperature, etc. (Foyer and Noctor, 2003).

In addition to scavenge reactive oxygen species (ROS), AsA is also involved in regulating photosynthesis through stomatal or non-stomatal factors (Davey et al., 2000; Smirnoff, 2005). In the present study, exogenous application of AsA through the rooting medium enhanced photosynthetic rate (A) in both cultivars under both non-saline and saline conditions. However, AsA-induced increase in A of the salt stressed plants of MH-97 was lower as compared to that in S-24 plants. It was also observed that root applied AsA caused an increase in stomatal conductance coupled with an increase in sub-stomatal CO_2 (C_i) of the salt-stressed plants of cv. S-24 indicating that A was mainly controlled by stomatal factors in S-24. This view is supported by an evidence that AsA has an important role in stomatal regulation (Chen and Gallie, 2004). These reports and the results of the present study suggested that changes in photosynthesis were due to stomatal limitations. However, in the salt-stressed plants of MH-97, AsA-induced changes in A were not accompanied by changes in g_s or C_i, indicating that changes in photosynthesis in MH-97 due to interactive effects of salinity and AsA may have been due to some non-stomatal factors. Such differential responses of both cultivars to AsA under saline conditions may have been due to differences in the protective mechanisms against salt stress. Similar differential protective mechanisms of photosynthesis in plants under salt stress have already been observed in different cultivars of barley (Salama et al., 1994), and wheat (Raza et al., 2006).

Maintenance of water homeostasis is necessary for various biochemical and physiological processes. With the change in stomatal conductance and transpiration rate under saline conditions, it is expected that plant water status may change. Although root applied AsA did not counteract the salt-induced reduction in plant water status (data for leaf water relation not shown), a positive association of leaf turgor with stomatal conductance and photosynthesis ($r = 0.448^{***}$ and 0.549^{***}) and negative association between leaf water potential with stomatal conductance and photosynthesis ($r = -0.707^{***}$ and -0.763^{***}) have been found, which indicates that AsA-induced non-significant changes in water relations may even cause changes in photosynthesis via stomatal limitations. Thus, it is likely that the osmotic effects (through ABA) or toxic effects of salt stress (displacement of K^+ by Na^+ in leaf epidermis caused oxidative stress) on certain metabolisms in guard cells are associated with changes in stomatal regulation (Dennison et al., 2001; Golldack et al., 2003; Pilot et al., 2003) and consequently with photosynthesis. If we draw the parallels between stomatal conductance and leaf Na^+ or leaf K^+, it is clear that stomatal conductance is positively associated with K^+ but the reverse was true with leaf Na^+ accumulation. Furthermore, exogenously applied AsA increased leaf K^+ and reduced K^+/Na^+ ratio in the roots (relatively more partitioning of Na^+ in the roots). These results support the findings of Al-Hakimi and Hamada (2001) in which it was reported that AsA application decreased Na^+ and increased K^+ in the shoots of salt-stressed wheat seedlings.

Calcium (Ca^{2+}) accumulation was increased in the leaves of salt-stressed plants of both cultivars when 50 or 100 mg L^{-1} AsA was applied. An increase in cytosolic Ca^{2+}, as a second messenger, might induce further physiological responses including expression of osmotic responsive genes (Pardo et al., 1998), and antioxidant enzymes (Chen and Li, 2001; Agarwal et

al., 2005). 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 plants, and maintenance of ion homeostasis (Mittler, 2002).

Overall, exogenous application of AsA increased endogenous level of AsA and CAT which had a protective effect on growth and photosynthetic capacity of wheat against salt-induced oxidative stress. Exogenous AsA application enhanced the stomatal conductance thereby favoring higher assimilation of CO_2, but this effect was cultivar specific. Exogenous application of AsA increased K^+ accumulation in the leaves and that of Na^+ in the roots. Although endogenous level of AsA played an important role in stomatal regulation and photosynthetic capacity, the detailed mechanism of AsA-induced enhanced in photosynthesis via stomatal regulations needs to be elucidated.

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