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Exogenous Application of Salicylic Acid through Rooting Medium Modulates Ion Accumulation and Antioxidant Activity in Spring Wheat under Salt Stress

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ABSTRACT

An experiment was conducted to assess whether exogenous application of salicylic acid through hydroponic culture could alter the ion concentrations and activate some antioxidative enzymes (superoxide dismutase, catalase & peroxidase) of a salt tolerant line (S-24) and a moderately salt sensitive cultivar (MH-97) of wheat. Seeds of cultivar were germinated in 0 or 150 mM NaCl in Hoagland's nutrient solution containing different levels of salicylic acid (SA) (0, 0.25, 0.50, 0.75 & 1.00 mM) for 7 d. Seven-days old wheat seedlings were transferred to hydroponics and grown at 0, or 150 mM NaCl for further 30 days. After 30 d, S-24 performed better than MH-97 under saline conditions with respect to ion accumulation and antioxidant activities. Exogenous application of SA promoted the activities of antioxidative enzymes and proline accumulation and ameliorated the adverse effects of salinity on growth of S-24, whereas there was no improvement in the salt sensitive cultivar MH-97. Of the varying SA levels used, the most effective levels for promoting antioxidant activity and ion accumulation were 0.25 and 0.75 mM under normal and saline conditions, respectively. The improvement in salinity tolerance due to SA application was associated with enhanced Na^+, Ca^{2+} and Cl^- uptake and antioxidative enzymes activities.

Key Words: Antioxidant enzymes; Ion accumulation; Free proline; Growth; Wheat

INTRODUCTION

Many Plant growth regulators play an important role in regulating the growth under stressful environment. Salicylic acid (SA) is a common plant-produced phenolic compound, which can function as growth regulator (Aberg, 1981). In addition, salicylic acid could be included in the category of phytohormones (Raskin, 1992). Exogenous application of SA may influence a range of processes in plants, including antioxidative enzyme activity (Almagro *et al.,* 2009), seed germination (Cutt & Klessing, 1992; Afzal *et al.,* 2006; Basra *et al.,* 2007), stomatal closure (Larque-Saavedra, 1979), ion uptake and transport (Harper & Balke, 1981), membrane permeability (Barkosky & Einhelling, 1993), disease resistance (Park *et al.,* 2009) and photosynthetic and growth rate (Khan *et al.,* 2003).

Salicylic acid plays an important role in the plant response to adverse environmental conditions, such as salt and osmotic stresses (Borsani *et al.,* 2001; Arfan *et al.,* 2007). It also provides protection in maize plants against low-temperature stress (Janda *et al.,* 1999; Kang & Saltveit 2002; Tasgin *et al.,* 2003). Salicylic acid also provides protection to barley seedlings against heavy metals stress (Metwally *et al.,* 2003) and to wheat plants against drought stress (Singh & Usha, 2003). In a study, SA increased the cytosolic Ca^{2+} concentration in tobacco suspension culture (Kawano & Muto, 2000), which, as second messenger, might induce further physiological responses including the expression of osmotic responsive genes for salt adaptation in plants (Pardo *et al.,* 1998). Salicylic acid induced free proline protects membranes and proteins against the adverse effects of inorganic ions in basil and marjoram (Fatma, 2007). Yet in another study, SA limited absorption and translocation of Na^+ and Cl^- and increased K^+ accumulation and translocation in Chinese spring wheat.

It is suggested that salicylic acid increases salt tolerance in wheat (Zhang *et al.,* 1998). SA treatment induced an increase in the content of soluble proteins and proline in barley seedlings (El-Tayeb, 2005). This suggested the involvement of SA in realization of different antistress programs. The objective of the present study was to investigate whether SA could alter various physiological/biochemical attributes such as ionic relations and activities of antioxidative enzymes in wheat plants under salt stress.

MATERIALS AND METHODS

A novel approach of exogenous application of SA was used to ameliorate the adverse effects of salt stress on spring wheat (*Triticum aestivum* L.) plants. Hence, the present study provides important information regarding

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physiological and biochemical roles of exogenously applied SA in salt tolerance of wheat. The study was conducted in the wirehouse of the Department of Botany, University of Agriculture, Faisalabad, Pakistan (latitude 31°30'N, longitude 73°10'E & altitude 213 m), with 10/14 light/dark period at 800–1100 mmol m^{-2} s^{-1} PPFD, a day/night temperature cycle of 26/15°C and 65±5% relative humidity. The seeds of line S-24 and cv. MH-97 were surface sterilized with 5% sodium hypochlorite for 5 min and then thoroughly rinsed with distilled water before further experimentation. Seeds of both genotypes were sown about 4 cm deep in thoroughly washed sand in 20 plastic pots (12 x 12 x 7 cm) with drainage holes in the bottom. The plants were supplied with 200 mL of half strength Hoagland's nutrient solution (Epstein, 1972). Salt treatments in this solution were begun from the start of the experiment. The NaCl treatments used were 0 and 150 mM in full strength Hoagland's nutrient solution. Seven days old seedlings were transferred in plastic containers (45 x 66 x23 cm) containing 20 L of half strength Hoagland's nutrient solution supplemented with or without salicylic acid (0, 0.25, 0.50, 0.75 & 1.00 mM in the rooting medium) under non-saline (0 mM NaCl) or saline conditions (150 mM NaCl). The experimental design was completely randomized (CRD) with four replicates (six seedlings per replicate of each cultivar). The nutrient solution was replaced weekly. All treatment solutions were continuously aerated. Determination for various physiological and biochemical attributes was made after 30 d.

Determination of ions (K^+, Ca^{2+}, Na^+ & Cl^-). Dried ground leaf or root material (0.1 g) was taken in each digestion tube and 2 mL of concentrated H_2SO_4 were added and then incubated it overnight at room temperature. Then 0.5 mL of H_2O_2 (35%) was poured down the sides of the digestion tube, ported the tubes in a digestion block and heated at 350°C until fumes were produced and continued to heat for another 30 min. The digestion tubes from the block were removed and cooled. Then 0.5 mL of H_2O_2 was slowly added and placed the tubes back into the digestion block. The above step was repeated until the cooled digested material was colorless. The volume of the extract was maintained up to 50 mL in volumetric flasks. The extract was filtered and used for K^+, Ca^{2+} and Na^+. The potassium (K^+), (Na^+) and calcium (Ca^{2+}) contents were determined with a flame spectrophotometer (Jenway PFP 7). Cl^- was determined by simple water extraction. Dried ground leaf or root material (0.1 g) was taken in a digestion tube and 10 mL of distilled water were added and then incubated it overnight at room temperature. Then ported the tubes in a digestion block and heated at 80°C until the volume in the test tubes remained half of the original volume. The digestion tubes from the block were removed and cooled. Then distilled water was added in each test tube to maintain volume up to 10 mL again and then the Cl^- concentration was determined with a chloride analyzer (Model 926, Sherwood Scientific Ltd., Cambridge, UK).

Free proline determination. The proline content in the 3rd fresh leaf was estimated according to the method of Bates *et al.* (1973). Proline concentration was computed from a standard curve and calculated on fresh weight basis as follows:-

μmole proline g^{-1} fresh weight = (μg proline mL^{-1} x mL of toluene/115.5)/(g of sample).

Total soluble proteins determination. The total soluble protein content in the 3rd fresh leaf was determined according to the method of Bradford *et al.* (1976).

Extraction and determination of antioxidant activities. Fresh leaves (0.5 g) were ground using a tissue grinder in 5 mL of 50 mM cooled phosphate buffer (pH 7.8) placed in an ice bath. The homogenate was centrifuged at 15000 *g* for 20 min at 4°C. The supernatant was used for assays of activities of enzymes.

The activity of superoxide dismutase (SOD) was determined by measuring its ability to inhibit the photoreduction of nitroblue tetrazolium (NBT) following the method of Giannopolitis and Ries (1977). The reaction solution contained 50 µM NBT, 1.3 µM riboflavin, 13 mM methionine, 75 nM EDTA, 50 mM phosphate buffer (pH 7.8) and 20 to 50 µL enzyme extract in a test tube. Test tube containing the reaction solution was irradiated under light (15 fluorescent lamps) at 78 µmoL m^{-2} s^{-1} for 15 min. The absorbance of the irradiated solution at 560 nm was read using a spectrophotometer (IRMECO U2020). One unit of SOD activity was defined as the amount of enzyme that inhibited 50% of NBT photoreduction.

Activities of catalase (CAT) and peroxidase (POD) were measured using the method of Chance and Maehly (1955) with some modification. The CAT reaction solution contained 50 mM phosphate buffer (pH 7.0), 5.9 mM H_2O_2, and 0.1 mL enzyme extract. Then reaction was initiated by adding enzyme extract. Changes in absorbance of the reaction solution at 240 nm were read every 20 s. One unit CAT activity was defined as absorbance change of 0.01 units per min. The POD reaction solution 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^{-1}. The activity of each enzyme was expressed on protein basis. Protein weight concentration of the extract was measured by the method of Bradford (1976).

Statistical analysis. Analysis of variance of data for all the parameters was computed using the MSTAT-C computer package (CoHort Software, Berkeley CA). The least significant differences between the mean values were calculated (Snedecor & Cochran, 1980).

RESULTS

Accumulation of Na^+ in shoots of both cultivars was significantly increased ($P<0.001$) with an increase in

growth medium salinity (Fig. 1). Line S-24 accumulated more Na^+ in shoots than MH-97 under saline condition. Exogenous application of SA did not affect the accumulation of Na^+ in shoots of both cultivars under non-saline conditions. However, exogenous application of SA caused an increase in accumulation of Na^+ in shoots of MH-97 under saline conditions (Fig. 1). In salinized S-24 plants, addition of 0.25 mM SA followed by 0.50 mM SA caused a significant reduction (*P<0.001*) in accumulation of Na^+ in shoot. Imposition of salt stress significantly increased the accumulation of Na^+ in the roots of both cultivars (Fig. 1). However, cultivars did not differ much for root Na^+ under either condition. Application of 0.75 mM SA caused a significant increase in root Na^+ of both cultivars under non-saline conditions (Fig. 1). However, addition of 0.50 mM and 1.00 mM SA caused a maximum reduction in root Na^+ of salinized S-24 and MH-97 plants, respectively.

Accumulation of K^+ in the shoots of both cultivars was significantly reduced (*P<0.001*) due to salt stress. Cultivar MH-97 was significantly higher in shoot K^+ than in S-24 under non-saline or saline conditions (Fig. 1). Addition of varying levels of SA did not affect the accumulation of K^+ in the shoots of both cultivars under both conditions except at 1.00 mM SA, where shoot K^+ was significantly reduced in MH-97 under both conditions. Imposition of salt stress caused a significant reduction (*P<0.001*) in root K^+ of both cultivars (Fig. 1). Increasing levels of SA caused a significant reduction (*P<0.001*) in root K^+ in MH-97 under salinity stress, particularly at the highest SA level (1 mM). In addition, the same was true for line S-24 under non-saline conditions.

Accumulation of Ca^{2+} in the shoots of both cultivars decreased significantly (*P<0.001*) due to salt stress (Fig. 2). Although difference between the cultivars was statistically non-significant, S-24 was higher in shoot Ca^{2+} than MH-97 under non-saline or saline conditions. Application of 0.25 mM SA caused a significant increase (*P<0.001*) in shoot Ca^{2+} of both cultivars under either condition. However, further increase in SA level caused a consistent reduction in shoot Ca^{2+} of both cultivars under non-saline conditions (Fig. 2). However, higher levels of exogenous SA did not affect the shoot Ca^{2+} of salinized plants of both cultivars. Root Ca^{2+} was significantly lesser in salinized plants of both cultivars in comparison with their respective controls. Both cultivars did not differ significantly (*P<0.001*) under either condition (Fig. 2). Root Ca^{2+} in both cultivars was decreased significantly (*P<0.001*) with an increase in external SA level under salinity stress.

Shoot Cl^- was significantly increased (*P<0.001*) in both cultivars due to salt stress, but the difference between them was not consistent at varying levels of SA. Addition of SA in the rooting medium caused a significant increase (*P<0.001*) in shoot Cl^- of both cultivars under both non-saline and saline conditions. However, such an effect was more pronounced in salinized plants of both cultivars, particularly at 0.75 and 1 mM SA levels (Fig. 2). Root Cl^- of

Fig. 1. Na^+ and K^+ of shoots and roots of two wheat cultivars differing in salinity tolerance when plants were grown hydroponically to varying concentrations of salicylic acid under saline or non-saline conditions

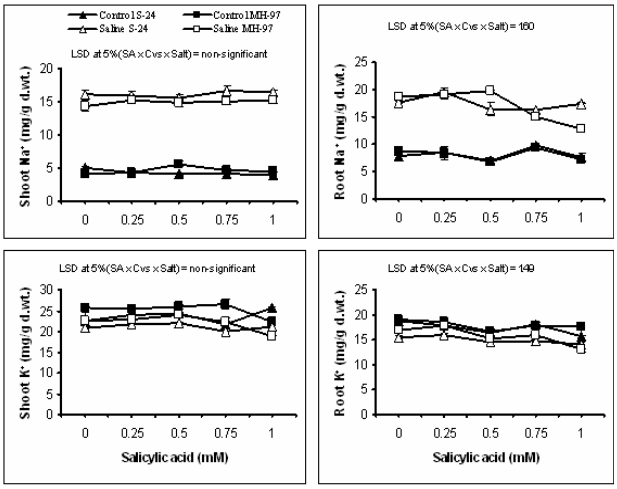
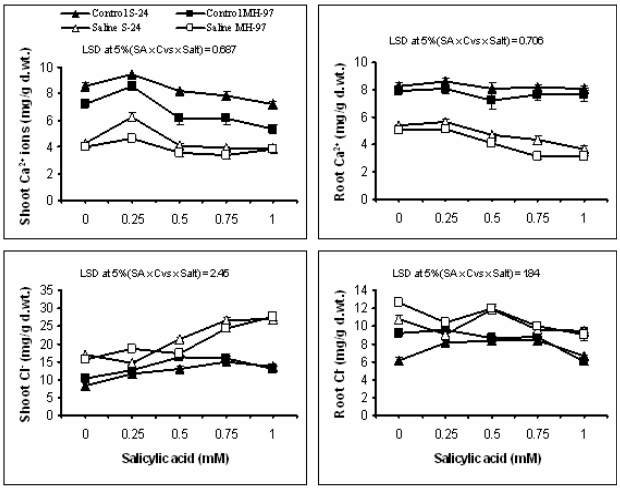


Fig. 2. Ca^{2+} and Cl^- of shoots and roots of two wheat cultivars differing in salinity tolerance when plants were grown hydroponically to varying concentrations of salicylic acid under saline or non-saline conditions



both cultivars was significantly increased (*P<0.001*) due to salt stress (Fig. 2). Exogenous application of SA particularly higher levels (0.75 & 1.00 mM) caused a significant reduction (*P<0.001*) in root Cl^- of MH-97 under both non-saline and saline conditions. Although exogenous application of SA significantly altered root Cl^- of non-salinized or salinized S-24 plants, the pattern of increase or decrease was not consistent with increase in exogenous SA level (Fig. 2).

Activity of CAT significantly increased (*P<0.001*) in both cultivars due to salt stress (Fig. 3). Exogenous application of SA significantly changed the CAT activities of both cultivars. Overall there was an increase in the

activities of CAT in S-24 with the application of varying levels of SA under both saline and non-saline conditions but the maximum increase in CAT activity was observed in S-24 at 0.25 mM SA level under both non-saline and saline conditions. In contrast, in MH-97, exogenous SA application did not markedly changed CAT activity under both conditions except at 1 mM SA level, where it caused a marked reduction (*P<0.001*) in CAT activity of MH-97 under non-saline conditions. However, application of 1.00 mM SA caused a significant increase in CAT activity of MH-97 under saline conditions. Overall the activities of S-24 were higher as compared to MH-97 at all exogenous SA levels.

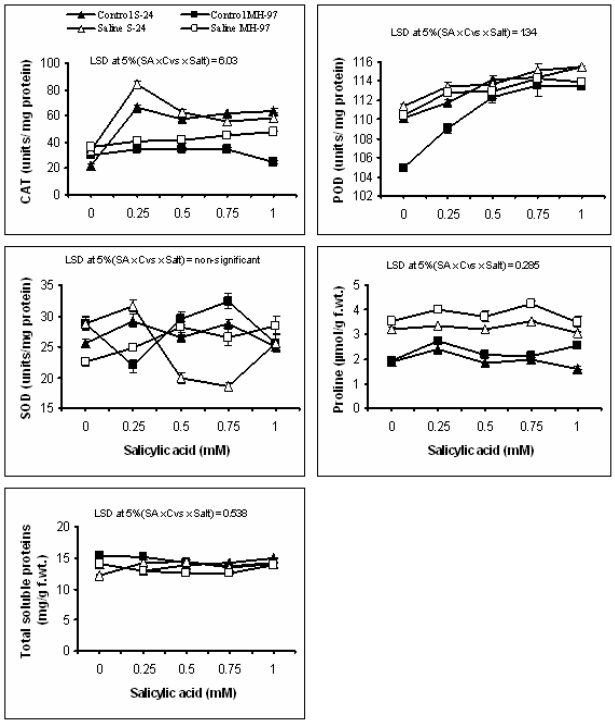
Imposition of salt stress significantly (*P<0.001*) increased POD activity of MH-97, whereas it did not affect that of S-24. However, POD activity of MH-97 was lower than that of S-24 under non-saline conditions (Fig. 3). Addition of varying levels of SA to the rooting medium caused a significant increase (*P<0.001*) in POD activity of both cultivars under both non-saline and saline conditions, but the maximum increase was observed in non-salinized plants of MH-97.

Saline growth medium caused a significant (*P<0.001*) effect on SOD activities in both cultivars. Salt stress caused a reduction in SOD activities of MH-97, whereas the reverse was true for S-24. Application of exogenous SA affected SOD activities of both cultivars. However, the pattern of increase or decrease in SOD activities of non-salinized plants of both cultivars was non-consistent with increase in external SA level. Under saline conditions, addition of 0.50 and 0.75 mM SA caused a significant decrease (*P<0.001*) in SOD activities of S-24 plants, whereas application of 0.50 mM SA caused an increase in SOD activity of MH-97 plants under salinity stress (Fig. 3).

Leaf proline was significantly (*P<0.001*) higher in salinized plants of both cultivars when compared with their respective controls (Fig. 3). Cultivar MH-97 had higher leaf proline contents than S-24 either condition. A significant increase (*P<0.001*) in leaf proline of non-salinized S-24 was observed at 0.25 mM SA level, whereas leaf proline of S-24 remained almost un-changed at all levels of SA under salinity. However, in MH-97, leaf proline was significantly increased due to exogenous supply of 0.25 and 1 mM SA under salt stress. In addition, maximum leaf proline was found in MH-97 plants supplied with 0.75 mM SA followed by those supplied with 0.25 mM SA.

Salt stress significantly (*P<0.001*) decreased leaf soluble proteins of both cultivars. Cultivar MH-97 had higher leaf soluble proteins than S-24 under non-saline or saline conditions. Application of SA caused a significant increase (*P<0.001*) in leaf soluble proteins in MH-97 under non-saline or saline conditions, whereas the reverse was true for S-24 under non-saline or saline conditions. A maximum reduction in leaf soluble proteins of MH-97 was found at 0.75 mM SA under both conditions (Fig. 3). However, a maximum increase in this attribute of S-24 was observed at

Fig. 3. Catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), proline and total soluble protein of two wheat cultivars differing in salinity tolerance when plants were grown hydroponically to varying concentrations of salicylic acid under saline or non-saline conditions



1 mM and 0.50 mM SA level under non-saline and saline conditions, respectively.

DISCUSSION

It is evident from the present results that the inhibitory effect of salt stress was more pronounced on MH-97 than on S-24. The results reported here for tissue ion concentration indicated an increase in accumulation of Na^+ and Cl^- coupled with a decrease in K^+ and Ca^{2+} in both cultivars. This is in agreement with the view that plants growing under saline conditions suffer ionic imbalance, nutrient deficiency and specific ion toxicity (Ashraf, 1994 & 2004; Munns *et al*., 2002 & 2005). However, SA application had no increasing or decreasing effect on accumulation of Na^+ and K^+ in both shoots and roots of both cultivars, which is contradictory to the recent findings of El-Tayyeb (2005) for salt stressed shoots of barley. In contrast, Ca^{2+} accumulation was increased in the shoots at 0.025 mM SA application and decreased in the roots of both cultivars under saline conditions. Kawano and Muto (2000) found that SA increased the cytosolic Ca^{2+} concentration in tobacco cell suspension culture. Such an increase is Ca^{2+} is implicated as a second messenger in inducing expression of osmotic responsive genes (Pardo *et al.,* 1998) and antioxidant

enzymes (Chen & Li, 2001; Agarwal *et al.,* 2005). In addition, SA application had a stimulatory effect on accumulation of Cl^- in the shoots of both cultivars and decreased accumulation of Cl^- in the roots.

Salt stress disturbs the ion homeostasis resulting in osmotic stress and ion toxicity effects; both cause generation of reactive oxygen species (ROS), which trigger phytotoxic reactions such as lipid peroxidation, protein degradation and DNA mutation (Alscher *et al.,* 1997; McCord & Fridovich, 2000; Mittler, 2002). To overcome salt-mediated oxidative stress, plants detoxify ROS by up-regulating antioxidant enzymes, like superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT). In view of a number of studies, salt tolerance is often correlated with a more efficient oxidative system (Mittova *et al.,* 2002; Bor *et al.,* 2003; Sawada *et al.,* 2009). Increase in SA-induced SOD activity with an increase in shoot Ca^{2+} at 0.25 mM SA in salinized S-24 plants supported the view that if sufficient Ca^{2+} is present, it acts as a second messenger and causes a transient increase in H_2O_2, which in turn induces antioxidant enzymes leading to decrease in ROS on long term basis (Chen & Li, 2001).

The pivotal role of Ca^{2+} in antioxidant enzyme signal transduction has been emphasized in experiments involving exogenous application of 5 mM Ca^{2+} through the rooting medium was more effective in increasing SOD, ascorbic acid peroxidase (APX) and CAT activities and in over-expressing Mn-SOD and Cu/Zn-SOD (Agarwal *et al*., 2005). The H_2O_2 produced by SOD is scavenged by CAT and a variety of POD (Mittler, 2002). In the present study, both CAT and POD activities enhanced by SA application suggest that ROS were effectively scavenged in both wheat cultivars. El-Tayyeb (2005) has suggested that SA-induced increase in activities of antioxidant enzymes is responsible for salt stress tolerance in barley. The data for SOD, CAT and POD reported here support this argument in view of the positive relationship between ion accumulation and praline contents and these antioxidant enzymes. However, enhancement or reduction in SA-induced SOD activity coupled with an increase in CAT and POD activities in salt stressed S-24 plants can be related to the findings of Yang *et al*. (2004) in which it was shown that high endogenous SA level in rice acted as an antioxidant to scavenge ROS and/or SA indirectly modulated redox balance through activation of antioxidant pathways to protect cells from oxidative damage caused by ROS (Oberle *et al.,* 1998; Castagen *et al.,* 1999). However, SA-induced enhancement in SOD activity coupled with an increase in CAT and POD activities in salt stressed plants of MH-97 was not effective in enhancing salt tolerance of the cultivar. These results suggest that salt tolerance of S-24 is not caused by SOD-CAT-POD antioxidant system and thus it supports the view that efficient antioxidant system is not necessarily involved in enhancing salinity tolerance in plants (Cavalcanti *et al.,* 2004).

Exogenous application of 0.25 and 0.75 mM SA increased the leaf proline in salt stressed MH-97, which is similar to the findings of Shakirova *et al*. (2003) in which it was found that SA application increased the proline in wheat seedlings. An increase in leaf proline with a decrease in total soluble proteins when 0.25 or 0.75 mM SA applied to salinized MH-97 plants support the view that SA stimulates the hydrolysis of soluble proteins, providing a pool of compatible osmolytes, which is important in osmotic adjustment in the presence of Na^+ (Ashraf & Harris, 2004). Proline was also increased in non-salinized MH-97 plants supplied with SA. Furthermore, leaf proline in MH-97 was higher than in S-24 at all levels of SA applications under saline conditions. However, salt stress or SA-induced high accumulation of proline in MH-97 plants as compared to S-24 cannot be related to its salt tolerance. These results can be related to those of Tal *et al*. (1979) who reported more proline accumulation in salt sensitive species of tomato than its tolerant wild relatives. In a study, Ashraf (1989) reported a negative relationship between proline accumulation and salt tolerance in *Vigna mungo*. Furthermore, Colmer *et al*. (1995) did not find any significant role of proline in salt tolerance in a wheat x *Lophopyrum elongatum* amphiploid. In view of all these reports as well as of the present study, it can be suggested that proline accumulation in both cultivars may not have a role in SA-induced improvement in salinity tolerance in both these wheat cultivars.

Our experiment indicates that salt induced inhibition in activities of antioxidant enzymes was found to be mitigated by the exogenous application of SA in both wheat cultivars. SA-induced greater accumulation of Ca^{2+} may have a role in up-regulating antioxidant enzymes.

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