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Acta Agriculturae Scandinavica Section B – Soil and Plant Science

ISSN: 0906-4710 (Print) 1651-1913 (Online) Journal homepage: https://www.tandfonline.com/loi/sagb20

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To cite this article: Aria Dolatabadian , Seyed Ali Mohammad Modarres Sanavy & Mozafar Sharifi (2009) Effect of salicylic acid and salt on wheat seed germination, Acta Agriculturae Scandinavica Section B – Soil and Plant Science, 59:5, 456-464, DOI: 10.1080/09064710802342350

To link to this article: https://doi.org/10.1080/09064710802342350

Published online: 10 Aug 2009.

Acta Agriculturae Scandinavica Section B – Soil and Plant Science, 2009; 59: 456–464

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ORIGINAL ARTICLE

Effect of salicylic acid and salt on wheat seed germination

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Abstract
The effects of pretreatment with salicylic acid on wheat seed germination (*Triticum aestivum* L. cv. Roshan), lipid peroxidation, and superoxide dismutase, catalase, polyphenol oxidase, and peroxidase activity were studied under conditions of salt stress. Seeds treated with different concentrations of salicylic acid were used for measuring germination traits. Salt stress was induced by sodium chloride solution. Seeds were soaked in salicylic acid solution for 24 h, dried with sterile paper, transferred to sterile Petri dishes, and treated with 10 ml NaCl solution at different concentrations. After 1 week, the number of germinated seeds, root length, seedling length, and dry weight were recorded. Antioxidant enzyme activity and lipid peroxidation were also assayed. Salinity decreased seed germination. Thus, a high concentration of NaCl (200 mM) decreased germination by 17.6% compared with control treatment. Salicylic acid significantly increased germination in stressed and control seeds. Salicylic acid increased the level of cell division of seedlings and roots, which increased plant growth. Salt stress significantly increased the activity of the antioxidative enzymes catalase, superoxide dismutase, peroxidase, and polyphenol oxidase in wheat seedlings, and salicylic acid reduced the activity of antioxidant enzymes as stress signal molecules. Our results indicated that scavenging of reactive oxygen species was effective, especially by salicylic acid, and that membrane damage was limited. The aim of the present work was to study the character of changes in enzymatic systems induced by NaCl and salicylic acid in wheat seedlings under conditions of salt stress. In brief, salicylic acid treatment reduced the damaging action of salinity on embryo growth and accelerated a restoration of growth processes; thereupon it may be effective for the improvement of seed germination in arid and semi-arid regions.

Keywords: *Antioxidant enzymes, lipid peroxidation, reactive oxygen species, salinity,* Triticum aestivum.
Abbreviations*:* CAT, catalase; MDA, malondialdehyde; POX, peroxidase; PPO, polyphenol oxidase; ROS, reactive oxygen species; SA, salicylic acid; SOD, superoxide dismutase.

Introduction

In both nature and agricultural systems, plants are exposed to ever-changing environmental conditions. They frequently must withstand drought, temperature extremes, and high salinity. To survive these challenges, plants have developed adaptive mechanisms that manifest themselves in morphological, physiological, developmental, and molecular changes (Bray, 1997).

Photosynthesis is a key metabolic pathway in plants and is a target for salt stress. The abscisic acid produced in response to salt stress decreases turgor in guard cells and limits the CO_2 that is available for photosynthesis (Leung et al., 1994). Moreover, during water stress brought about by salt stress, a reduction of chloroplast stromal volume and the generation of reactive oxygen species (ROS) are also thought to play important roles in inhibiting photosynthesis (Price & Hendry, 1991). Reactive oxygen species can be generated in the chloroplast by direct transfer of excitation energy from chlorophyll to produce singlet oxygen, or by univalent oxygen reduction at photosystem I, in the Mehler reaction (Foyer et al., 1994). In particular, superoxide anions of the active oxygen species are generated if the dark reaction of photosynthesis is hindered by environmental stresses and the excessive light energy cannot

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(Received 8 May 2008; accepted 13 July 2008)

ISSN 0906-4710 print/ISSN 1651-1913 online
DOI: 10.1080/09064710802342350

be used for the reduction of $NADP^+$ in the ferredoxin of the chloroplast (Elstner, 1982). It is generally accepted that superoxide is converted into H_2O_2 by superoxide dismutase (SOD; EC 1.15.1.1) and then metabolized to water by peroxidase (POX; EC 1.11.1.7) and glutathione reductase in the chloroplasts. Furthermore, H_2O_2 that has diffused to the outer portion of the chloroplast might be detoxified to water by catalase (CAT; EC 1.11.1.6) in the leaf cells (Hausladen & Alscher, 1993). Therefore, it is crucial that plants maintain the activities of these enzymes in order to accommodate these oxidative stresses. It has been reported that the CAT of the ROS-scavenging system plays a decisive role in the salt tolerance of rice cultivars (Shim et al., 1999).

Salicylic acid (SA; 2-hydroxybenzoic acid) is an endogenous growth regulator of a phenolic nature, which participates in the regulation of physiological processes in plants. Salicylic acid is an important secondary signal in plants that plays a major role in the activation of defence genes in response to pathogen attack. Most signalling is buffered by the presence of multiple pathways (Glazebrook et al., 2003). Salicylic acid, for example, acts as a natural inductor of thermogenesis in Arum lily, induces flowering in a range of plants, and controls ion uptake by roots and stomatal conductivity (Raskin, 1992). Salicylic acid plays an important role in the defence response to pathogen attack in many plant species. Salicylic acid mediates the oxidative burst that leads to cell death in the hypersensitive response and acts as a signal for the development of systemic acquired resistance (Shirasu et al., 1997).

Several other studies also support a major role for SA in modulating the plant response to several abiotic stresses (Senaratna et al., 2000). The exogenous application of SA results in plant tolerance to many biotic and abiotic stresses including fungi, bacteria, viruses (Delany et al., 1994), chilling, drought, and heat (Senaratna et al., 2003). Treating mustard seedlings with exogenous SA improves their thermotolerance and heat acclimation (Dat et al., 1998). In maize plants, pretreatment with SA induces antioxidant enzymes, which in turn increase tolerance to chilling (Janda et al., 1999). Salicylic acid induces increased resistance to water deficit in tomatoes (Bezrukova et al., 2001) and to low and high temperature in bean plants (Senaratna et al., 2000). Salicylic acid is involved in resistance to stress from high temperatures, for instance, in pea plants (Liu et al., 2006). Salicylic acid alters key plant functions including nutrient uptake (Glass, 1975), membrane functioning (Glass & Dunlop, 1974), water relations (Barkosky & Einhellig, 1993), stomatal functioning (Aldesuquy et al., 1998), inhibition of ethylene biosynthesis (Srivastava & Dwivedi, 2000), and increased growth (Rajasekaran & Blake, 1999). These functions may play a key role in plant tolerance to salt stress.

Both salt and osmotic stress leads to oxidative stress and severe impairment of seedling survival. In the plant life cycle, the seed and seedling stages are key developmental stages that determine the final yield of crops. Both are very sensitive to environmental stresses (Koornneef et al., 2002).

The investigation reported herein was out to study the effect of grain-soaking with SA on the salt stress responses of wheat. We assumed that SA could prevent cell damage and neutralize ROS. Our results showed that SA was involved in the seedling response to salt stress, by changes in activity of antioxidant enzymes, such as CAT, SOD, polyphenol oxidase (PPO), and POX. We conclude that SA directly decreases the effects of salt stress, prevents increased antioxidant enzyme activity during germination, and increases salt tolerance.

Materials and methods

Plant material and growth conditions

Seeds of wheat (*Triticum aestivum* L. cv. Roshan) were surface-sterilized for 5 min in sodium hypochlorite solution and then in 96% ethanol for 30 s. After sterilization, seeds were soaked in 0, 0.5, or 1 mM SA for 24 h, and then incubated in 9-cm sterile Petri dishes on a single layer of filter paper moistened with 10 ml NaCl solution at 0, 50, 100, or 200 mM. To prevent infection and evaporation of solution, all of the plates were closed with parafilm. All operations were performed under laminar flow. The Petri dishes were incubated in a germinator at 25°C.

Seed germination and sampling

Numbers of germinated seeds were recorded after eight days. Total numbers of germinated seeds, length of root, length of seedlings, and their dry weight were recorded immediately after incubation was terminated. Seedlings were dried for 24 h in an oven at 50°C. The percentage of seeds that germinated was calculated. The remains of seedlings were frozen in liquid N_2 and stored at -80°C until biochemical analysis.

Extract preparation

Seedlings (0.2 g) were homogenized using a mortar and pestle with 3 ml ice-cold extraction buffer (25 mM sodium phosphate buffer, pH 7.8). The homogenate was centrifuged at 18 000 ***g*** for 30 min at 4 °C

and the supernatant was filtered through filter paper. The supernatant fraction was used as a crude extract for the assay of enzyme activity and protein content. All operations were carried out at 4°C.

Antioxidant enzyme assay

Catalase activity was estimated by the method of Cakmak and Horst (1991). The reaction mixture contained 100 µl crude enzyme extract, 500 µl 10 mM H_2O_2, and 1.4 ml 25 mM sodium phosphate buffer. The decrease in A_{240} was recorded for 1 min by spectrophotometer (model Cintra 6 GBC, manufactured in Australia), and enzyme activity of the extract was expressed as units per milligram of protein.

Superoxide dismutase activity was determined according to the method of Giannopolitis and Ries (1977). The reaction mixture contained 100 µl 1 µM riboflavin, 100 µl 12 mM l-methionine, 100 µl 0.1 mM EDTA (pH 7.8), 100 µl 50 mM Na_2CO_3 (pH 10.2), and 100 µl 75 µM nitroblue tetrazolium (NBT) in 2.3 ml 25 mM sodium phosphate buffer (pH 6.8), with 200 µl crude enzyme extract in a final volume of 3 ml. SOD activity was assayed by measuring the ability of the enzyme extract to inhibit the photochemical reduction of NBT. Glass test tubes that contained the mixture were illuminated with a fluorescent lamp (120 W). Identical tubes that were not illuminated served as controls. After illumination for 15 min, the absorbance was measured at 560 nm. Enzyme activity of the extract was expressed as units per milligram of protein.

Polyphenol oxidase (PPO; EC 1.10.3.1) activity was determined by the oxidation of catechol in the presence of H_2O_2. The increase in A_{410} was recorded for 1 min (Ghanati et al., 2002). The reaction mixture contained 100 µl crude enzyme, 500 µl 5 mM H_2O_2, 500 µl 0.02 mM catechol, and 1.9 ml 60 mM potassium phosphate buffer (pH 6.1). The enzyme activity of the extract was expressed as units per milligram of protein.

Peroxidase activity was determined by the oxidation of guaiacol in the presence of H_2O_2. The increase in A_{470} was recorded for 1 min (Ghanati et al., 2002). The reaction mixture contained 100 µl crude enzymes, 500 µµl 5 mM H_2O_2, 500 µl 28 mM guaiacol, and 1.9 ml 60 mM potassium phosphate buffer (pH 6.1). Enzyme activity of the extract was expressed as units per milligram of protein.

Determination of malondialdehyde levels in crude extracts

The level of membrane damage was determined by measuring malondialdehyde (MDA) as the end product of membrane lipid peroxidation (De Vos et al., 1991). Samples were homogenized in an aqueous solution of 10% trichloroacetic acid (w/v) and aliquots of the filtrates were heated at 100 °C for 30 min in 0.25% thiobarbituric acid. The amount of MDA was determined from A_{532}, followed by correction for nonspecific absorbance at 600 nm. The content of MDA was determined using the extinction coefficient of MDA (ε 155 μM^{-1} cm^{-1}).

Protein assay

Total protein content was determined using bovine serum albumin (BSA) as a standard, according to the method of Bradford (1976), using 1 ml Bradford solution and 100 µl crude extract. Coomassie Blue G 250 (100 mg) was dissolved in 50 ml methanol. The solution was added to 100 ml 85% H_3PO_4, and diluted to 200 ml with distilled water. The protein concentration was calculated from a BSA standard curve.

Statistical analysis

All data were analysed using SAS software (SAS Institute Inc., 1997). Each treatment was analysed in three replications. When analysis of variance (ANOVA) showed significant treatment effects, Duncan's multiple range test was applied to compare the means at $P<0.05$ (Steel & Torrie, 1980). The simple correlation among the parameters studied was also calculated.

Results

Salt stress significantly affected all the traits investigated in this study. In addition, SA also had a significant effect on these variables**.** Salt stress significantly decreased germination in the absence of SA (Figure 1). Increasing NaCl concentration led to a decrease in germination, and SA induced germination at all levels of salt stress. The highest germination percentage was observed in seedlings without salt stress and with 0.5 mM SA treatment. The lowest germination percentage was observed with 200 mM NaCl with 0 or 1 mM SA. The addition of 0.5 mM SA to 50 mM NaCl decreased the harmful effect of salt stress, and the germination percentage did not differ significantly from that in the presence of 0.5 mM SA without salt stress. Increasing SA concentration to 1 mM showed an inhibitory effect on germination for all treatments. Therefore, 0.5 mM SA had a positive effect on germination and inhibited the adverse effect of salt stress. This indicates that SA pretreatment increases salt tolerance in wheat seeds.

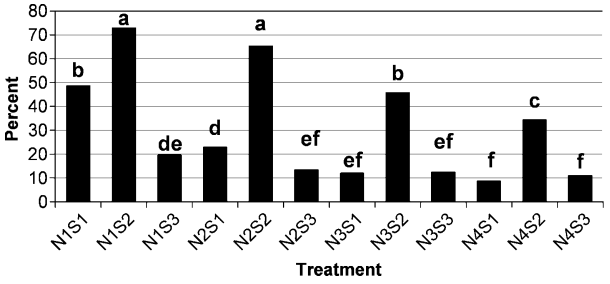

Figure 1. Changes in percentage of germination with different treatments of NaCl and SA. N_1, 0 mM NaCl; N_2, 50 mM NaCl; N_3, 100 mM NaCl; N_4, 200 mM NaCl. S1, 0 mM SA; S2, 0.5 mM SA; S3, 1 mM SA. All the values followed by the same letter in each column are not statistically different at the $P < 0.05$ probability level.

Dry weights of seedlings decreased progressively as salt stress increased (Figure 2). The greatest dry weight of seedlings was in plants without salt stress treated with 0.5 and 1 mM SA, 50 mM NaCl and 0.5 mM SA, and 100 mM NaCl and 0.5 mM SA. There was no significant difference in the dry weight of seedlings between these treatments. The increase in dry weight of seedlings was due to increased root and shoot growth. This means that 0.5 mM SA inhibited the effect of salt stress on dry weight of seedlings, but that it was ineffective with 200 mM NaCl. The lowest dry weight of seedlings was in plants treated with 200 mM NaCl and 1 mM SA.

Salicylic acid increased the length of shoots and roots in plants without salt stress (Figures 3 and 4). With salt stress, the only significant increase in shoot length was with 0.5 mM SA and 100 mM NaCl. Usage of 0.5 mM SA increased root length under all conditions of salt stress when compared with stress and without SA treatments.

Antioxidant enzyme activity was greatly affected by salt stress. Catalase activity showed the greatest increase in the presence of 200 mM NaCl without SA (Figure 5), and its minimum activity was observed with 50 mM NaCl and 0.5 mM SA. Salicylic acid at 0.5 mM decreased CAT activity in the presence of 100 and 200 mM NaCl, but it had

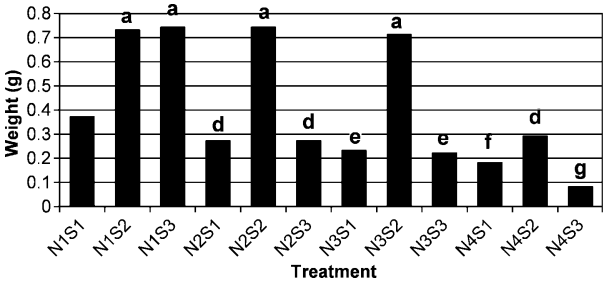

Figure 2. Changes in dry weight of seedlings with different treatments of NaCl and SA. All the values followed by the same letter in each column are not statistically different at the $P < 0.05$ probability level.

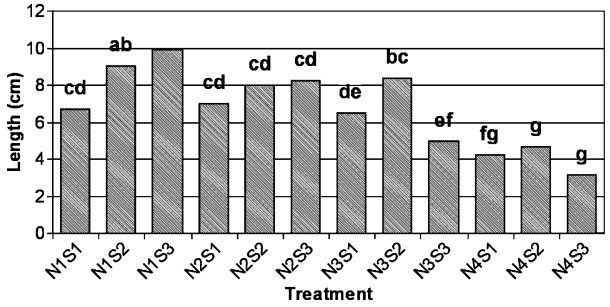

Figure 3. Changes in shoot length with different treatments of NaCl and SA. All the values followed by the same letter in each column are not statistically different at the $P < 0.05$ probability level.

no effect at 50 mM NaCl. An increase in SA concentration to 1 mM increased CAT activity.

Superoxide anions are generated in biotic and abiotic stress such as salt stress. SOD is an antioxidant enzyme that scavenges free radicals by converting them into H_2O_2. Superoxide dismutase activity increased significantly with increasing salinity (Figure 6). Salicylic acid at 0.5 mM decreased SOD activity in salt-stressed seedlings. The highest concentration of SA induced SOD activity.

Polyphenol oxidase activity was greatest with 200 mM NaCl without SA (Figure 7). PPO activity increased as salinity increased. Salicylic acid at 0.5 mM decreased PPO activity in the presence of 100 and 200 mM NaCl. Salicylic acid at 1 mM decreased PPO activity at only 200 mM NaCl, and was ineffective at other salinity levels.

The activity of POX, which is an enzyme involved in anti-oxidative defence mechanisms, increased progressively as salinity increased (Figure 8). The highest activity was observed at 200 mM NaCl without SA. Pretreatment with 0.5 mM SA decreased POX activity at all salt levels and none. Pretreatment with 1 mM SA also decreased POX activity in comparison with 0 mM SA, but increased it in plant under 0.5 mM SA treatment.

Salicylic acid at 0.5 or 1 mM significantly decreased the level of lipid peroxidation and

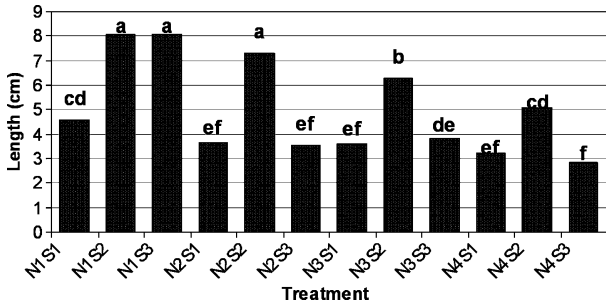

Figure 4. Changes in root length with different treatments of NaCl and SA. All the values followed by the *same letter in each column are not statistically different at the* $P < 0.05$ probability level.

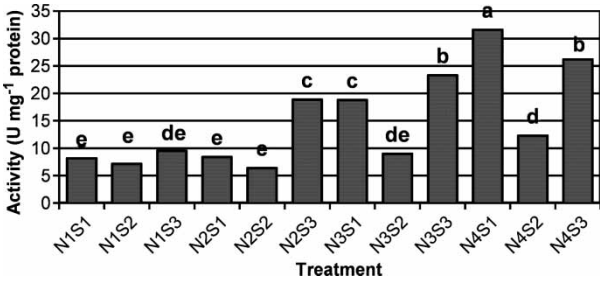

Figure 5. Changes in CAT activity in wheat seedlings treated with different concentrations of NaCl and SA. All the values followed by the same letter in each column are not statistically different at the $P < 0.05$ probability level.

electrolyte leakage in seeds subjected to salt stress of 200 mM NaCl (Figure 9). This indicated that SA treatment may have decreased the membrane damage caused by NaCl.

Salt stress, similar to other environmental stress, decreased protein content as the salinity level increased. Salicylic acid decreased protein content in the presence and absence of salt stress with 50 and 100 mM NaCl (Figure 10).

In our experiment, seed germination had a positive and significant correlation with seedling weight, shoot length, root length, and protein content (Table I). Results showed that seed germination has a negative and significant correlation with antioxidant enzyme activity and MDA content. Weight of seedlings also had a positive and significant correlation with root and shoot length and had a negative and significant correlation with antioxidant enzymes and MDA content. Correlation between shoot length and antioxidant enzymes activity and between shoot length and MDA was negative and significant at $P<0.01$. Similarly, results observed between root length with antioxidant enzymes activity and between root length with MDA content were also significant and negatively correlated. A strong positive correlation between MDA content and CAT, PPO, and POX has been found. In addition, protein and MDA content in seedlings showed a high and

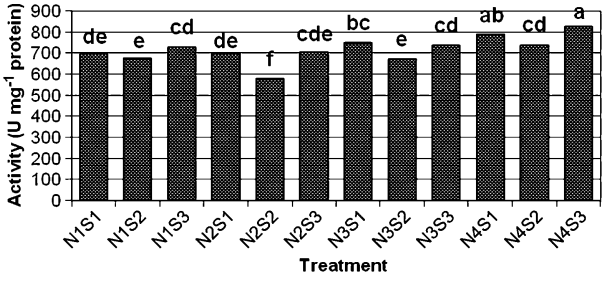

Figure 6. Changes in SOD activity in wheat seedlings treated with different concentrations of NaCl and SA. All the values followed by the same letter in each column are not statistically different at the $P < 0.05$ probability level.

Table I. Correlation analysis between traits of germination, enzyme activity, malondialdehyde and protein content of wheat seedlings exposed to salinity and salicylic acid.

Trait	Germination	Weight	Shoot length	Root length	Catalase	Superoxide dismutase	Polyphenol oxidase	Peroxidase	Malondialdehyde	Protein
Germination	1									
Weight	0.8452**	1								
Shoot length	0.4604**	0.6875**	1							
Root length	0.7149**	0.8546**	0.6818**	1						
Catalase	−0.7389**	−0.7152**	−0.6892**	−0.6911**	1					
Superoxide dismutase	−0.6919**	−0.7393**	−0.4861**	−0.5742**	0.4550**	1				
Polyphenol oxidase	−0.3544*	−0.3135ns	−0.4383**	−0.3412*	0.6253**	0.0105ns	1			
Peroxidase	−0.7730**	−0.7591**	−0.4684**	−0.6735**	0.6406**	0.4467**	0.6692**	1		
Malondialdehyde	−0.5519**	−0.4936**	−0.4893**	−0.4649**	0.6948**	0.2761ns	0.7799**	0.6325**	1	
Protein	0.4138*	0.1721ns	0.0733ns	0.0720ns	−0.5054**	0.1751ns	−0.7447**	−0.5518**	−0.5985**	1

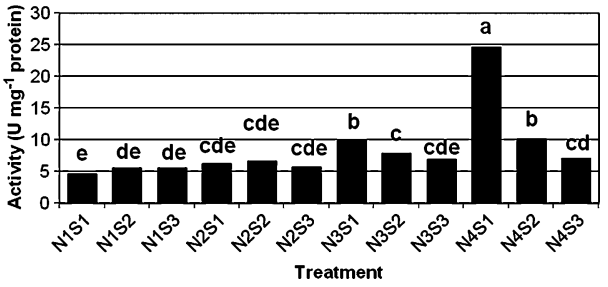

Figure 7. Changes in PPO activity in wheat seedlings treated with different concentrations of NaCl and SA. All the values followed by the same letter in each column are not statistically different at the $P < 0.05$ probability level.

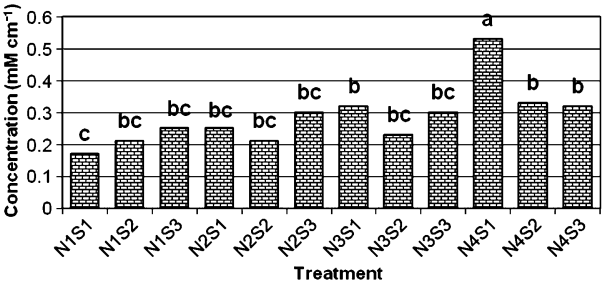

Figure 9. Changes in MDA content in wheat seedlings treated with different concentrations of NaCl and SA. All the values followed by the same letter in each column are not statistically different at the $P < 0.05$ probability level.

negative correlation ($P < 0.01$), which is an indication of the connection between the function of these compatible solutes (Table I).

Discussion

According to the above results, the percentage seed germination, seedling growth, and other germination traits decreased significantly as NaCl concentration increased. This agrees with the results of Ghoulam et al. (2001), who showed that salinity markedly reduces the growth (fresh and dry weight) of shoots and roots of sugar beet plants. Salicylic acid induced synthesis of protein kinases, which play an important role in regulating cell division, differentiation and morphogenesis (Chen et al., 1997).

This research showed that the exogenous application of SA increased seed germination and seedling growth under salinity conditions. The exogenous application of SA may influence a range of diverse processes in plants, including seed germination, ion uptake and transport, and membrane permeability. These results are consistent with those of Rajasekaran et al. (2002) and Shakirova et al. (2003), who showed that seed germination is promoted by the application of SA. Gutierrez-Coronado et al. (1998) have also reported a similar increase in the growth of shoot and roots of soybean plants under normal conditions in response to SA treatment. It has also been reported that SA increases the dry weight of wheat seedlings under water stress (Singh & Usha, 2003), which is consistent with our results for wheat seedlings. Salicylic acid increased the fresh and dry weight of shoots and roots of stressed maize plants (Khodary, 2004). Our results showed that pretreating wheat grains with SA results in an increase in salt tolerance.

Our results also showed that SOD and CAT activity were increased by salt stress and decreased by SA. It has been found that the inhibition of CAT, an H_2O_2-scavenging enzyme, by SA plays an essential role in the generation of ROS (Horváth et al., 2002). The reduction of CAT activity by SA might increase the accumulation of H_2O_2. H_2O_2 may be removed by CAT or by ascorbate peroxidase of the ascorbate–glutathione antioxidant cycle (Foyer et al., 1997). A changed pattern of antioxidants in the presence of abiotic stress under SA-primed and SA-free conditions has been reported in barley (Matewally et al., 2003). The observation that specific SA-binding activity found in tobacco (Chen et al., 1993a,b) and in several other plant species (Sanchez-Casas & Klessig, 1994) has CAT activity suggests that SA acts by enhancing ROS such as H_2O_2 (Chen et al., 1993a,b). The inhibition of CAT activity by SA *in vitro* (Chen et al., 1993a,b) provides support for this hypothesis. Given that treatment of

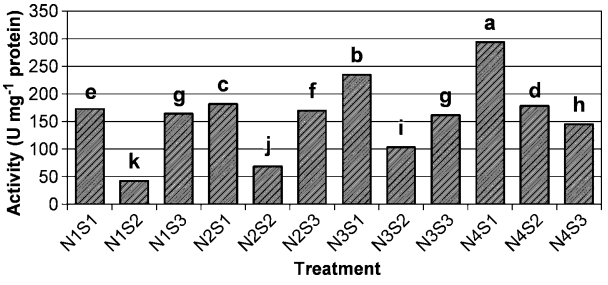

Figure 8. Changes in POX activity of wheat seedlings treated with different concentrations of NaCl and SA. All the values followed by the same letter in each column are not statistically different at the $P < 0.05$ probability level.

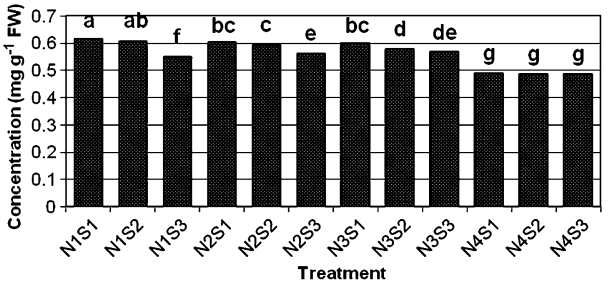

Figure 10. Changes in protein content of wheat seedlings treated with different concentrations of NaCl and SA. All the values followed by the same letter in each column are not statistically different at the $P < 0.05$ probability level.

seeds with H_2O_2 itself alleviates oxidative damage caused by salt stress in wheat plants (Wahid et al., 2007), it seems that SA may exert its protective effect partially through the transiently increased level of H_2O_2.

In this work, PPO and POX activity was increased by salt stress and decreased by SA. Salicylic acid has been shown to suppress total POX activity in wheat in the presence of pathogens (Maksimov et al., 2004). Decrease of POX and PPO activity can be due to scavenging of ROS by SA.

The data also showed that lipid peroxidation in seedlings increased as the stress level increased, but that soaking grains with SA led to a significant decrease in the level of lipid peroxidation in the salt-stressed seedlings. Under NaCl treatment, an increase in MDA content was indicative of oxidative stress in seedlings. However, MDA content was low in salt-stressed seedlings that were pretreated with SA, but most MDA was observed in plants treated with 200 mM NaCl without SA. Similarly, Tari et al. (2002) found a significant decrease in the concentration of thiobarbituric acid-reactive compounds in salt-stressed tomato plants in response to pretreatment with SA. The results of the present study are also consistent with those of Popova et al. (2003), who showed that pretreatment with SA decreases lipid peroxidation and electrolyte leakage in barley plants. The enhancement of MDA production and its subsequent lowering under SA treatment was further substantiated by the histochemical staining pattern of these seedlings. Similar results have been obtained in *Lemna minor* under NaCl stress (Panda & Upadhyay, 2004). Salt stress enhances free radical levels in plants. That being so, membrane damage was investigated by monitoring MDA content and electrolyte leakage. Treatment with NaCl resulted in an increased loss of membrane integrity. These results agree with those of Bor et al. (2003), who found that salt stress increases lipid peroxidation in the leaves of two beet species. This increase was particularly high with SA-free treatments. The reduction in membrane damage that is associated with increased dry weight of water-stressed seedlings in response to SA may be related to the induction of antioxidant responses that protect the plant from oxidative damage. Senaratna et al. (2000) have suggested that a similar mechanism is responsible for SA-induced multiple stress tolerance in bean and tomato plants. It seems that by quenching the destructive effect of free radicals, SA prevents lipid peroxidation and decreased MDA content. These free radicals can damage the structure of proteins and nucleic acid (Noctor & Foyer, 1998) and decrease protein content.

We found an interesting inverse correlation between the activities of antioxidant enzymes with germination and growth seedlings (Table I). Correlation between MDA and growth traits also was negative. Salt stress apparently promotes the accumulation of reactive oxygen species in plants and results in increased oxidative injuries. The results of the present study are also consistent with those of Dolatabadian et al. (2008), who showed that salt stress increases antioxidant enzyme activity and malondialdehyde content in canola plants. This suggests that seedlings growth decreased with higher ROS levels. These data also strengthen the role of antioxidant enzymes as an important scavenger during stress conditions.

Overall, the data presented indicate that pretreatment of wheat seeds with SA contributes to an increase in salt-stress resistance, which is a mediator of the protective action of SA.

Acknowledgement

The authors thank Dr. Faezeh Ghanati Associate Professor of Plant Science Department of Tarbiat Modares University for useful instruction in the laboratory.

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