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RESEARCH PAPERS

Salicylic Acid Affects Wheat Cultivars Antioxidant System under Saline and Non-Saline Condition[1]

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Abstract—Salicylic acid (SA), a naturally occurring plant hormone, is an important signal molecule known to have diverse effects on biotic and abiotic stress tolerance. In this study the effects of exogenous application of different levels of SA (0, 250, and 750 μM) in hydroponic culture on hydrogen peroxide (H_2O_2) and MDA generation, the content of soluble proteins and activities of antioxidative enzymes such as superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) in shoots of two wheat (*Triticum aestivum* L.) cultivars (Azar2 and Chamran) under different concentrations of NaCl (0, 75, and 150 mM) were investigated. Concentration of 150 mM NaCl induced deleterious effects in both wheat cultivars. Application of 250 μM SA was effective in alleviation of salt stress. Activities of SOD, POD, and CAT enzymes were augmented by SA treatments. The highest SOD and POD activities were recorded at 250 μM SA + 150 mM NaCl, while CAT activity was increased at 250 μM SA + 75 mM NaCl. With the increase in antioxidative enzyme activities, SA decreased H_2O_2 and MDA content of the seedlings grown under salt stress.

Keywords: *Triticum aestivum*, antioxidative enzymes, growth, H_2O_2, malondialdehyde, salicylic acid, salinity

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INTRODUCTION

Salinity is one of the most severe abiotic stresses in half of all irrigated lands, causing adverse effects at physiological, biochemical and molecular levels and limiting crop productivity [1, 2]. The deleterious effects of salinity on plant growth are associated with (1) low osmotic potential of soil solution (water stress), (2) nutritional imbalance, (3) specific ion effect (salt stress) or (4) a combination of these factors [3]. All of these stresses cause the formation of ROS and leading to oxidative stress [4]. Furthermore, plants possess a complex antioxidant defense system in order to reduce oxidative damage by detoxifying free radicals. The important enzymatic defense system includes superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT). SOD is a major scavenger of $O_2^{\cdot -}$ and its enzymatic action results in the formation of H_2O_2 and O_2. Catalase and a variety of peroxidases catalyze the breakdown of H_2O_2. Catalase, which is apparently absent in the chloroplasts, dismutates H_2O_2 into H_2O and O_2. Whereas peroxidase decomposes H_2O_2 by oxidation of co-substrates such as phenolic compounds and/or anti-oxidants [5].

Salinity causes the notable changes in the antioxidative enzymes activities and redox metabolism of different plant species. These changes dependend on plant species and cultivars. For example in salt tolerant cultivars of *Oryza sativa, Brassica juncea*, and *Morus nigra* the activities of CAT, POX, SOD, and glutathion reductase (GR) increased in response to salt stress, but in salt sensitive cultivars the activities of these enzymes were reduced [6, 7]. The levels of ascorbate and reduced glutathion were also higher in salt tolerant cultivars of rice in comparing to sensitive ones under salt stress [6].

Salicylic acid (SA) acts as an endogenous signal molecule in plants, and has diverse effects on tolerance to abiotic stresses [8, 9] such as ozone, UV-B, heat, heavy metals, and osmotic stress [10, 11]. Studies on different plants demonstrated that SA may be used to alleviate salt stress [10].

The role of antioxidative enzymes in mechanisms of tolerance developed in response to different environmental extremes such as salinity has already been demonstrated. Many studies have showed correlation between the resistance to environmental stresses and the efficiency of the antioxidant system. This work was conducted to study the antioxidant systems of two wheat cultivars in saline conditions and the role of different concentrations of SA in improvement of salt effects.

[1] The article is published in the original.

Abbreviations: CAT—catalase; POD—peroxidase; SOD—superoxide dismutase; SA—salicylic acid; FW—fresh weight.

MATERIALS AND METHODS

Plant material. The seeds of two different wheat cultivars (*Triticum aestivum* L. cvs. Chamran and Azar2) were obtained from the Agricultural Research Center of Tabriz, Iran.

Growth conditions. All experiments were conducted hydroponically in a growth chamber with a temperature regime of 28/20°C (day/night), 16/8 h (light/dark) period and relative humidity of 70%. Seeds were germinated in Petri dishes and transferred to plastic containers with 2 L of nutrient solution (50%) and pre-cultured for 5 days. 10-day-old plants were transferred to the full strength nutrient solution, containing 0 (control), 75, and 150 mM NaCl and three levels of SA including 0 (control), 250 μM (an appropriate concentration according to previous studies), and 750 μM (relatively high concentration). After 21 days of treatment, the plants were used for further analyses.

Enzyme assays. Fresh leaf samples were used for enzymes extraction and determination of protein and metabolites. Leaf samples were ground at 4°C in extraction buffer. Each enzyme assays were tested for linearity between the volume of crude extract and the measured activity. Changes in the absorbance of substrates or products were measured using spectrophotometer (Specord 200, Analytic Jena, Germany).

Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined according to Giannopolitis and Ries [12]. The enzyme was extracted in 25 mM Hepes (pH 7.8) and 0.1 mM EDTA, and centrifuged at 15000 *g* for 15 min. Test tubes containing 25 μL of enzyme extract, 25 μL extraction buffer and 450 μL of the reaction mixture were incubated at 22°C and a light intensity of 400 μmol/(m^2 s). The reaction mixture contained 25 mM Hepes (pH 7.6), 0.1 mM EDTA, 50 mM Na_2CO_3 (pH 10.2), 12 mM L-methionine, 75 μM NBT and 1 μM riboflavin. The reaction was started by removing a dark plastic foil from the surface of samples and continued for 10 min. One unit of SOD activity was defined as the amount of enzyme required to induce 50% inhibition of NBT reduction as measured at 560 nm, compared with control samples without enzyme aliquot.

Peroxidase (POD, EC 1.11.1.7) activity was determined using the guaiacol test [13, 14]. The enzyme was extracted by 10 mM phosphate buffer (pH 7.0) and assayed in solution contained 10 mM phosphate buffer, 5 mM H_2O_2, and 4 mM guaiacol. The reaction was started by addition of the enzyme extract at 25°C and was followed 2 min photometrically at 470 nm. The enzyme activity unit was calculated as amount of protein required for the formation of 1 μM tetraguaiacol for 1 min.

Catalase (CAT, EC 1.11.1.6) activity was assayed by monitoring the decrease in absorbance of H_2O_2 at 240 nm [13]. The enzyme was extracted in 50 mM phosphate buffer (pH 7.0). The assay solution contained 50 mM phosphate buffer and 10 mM H_2O_2. The reaction was started by addition of enzyme aliquot to the reaction mixture and the changes in absorbance were monitored for 2 min. Unit activity was taken as amount of enzyme, which decomposes 1 M of H_2O_2 in 1 min.

Other assays. Soluble proteins were determined as described by Bradford [15] using a commercial reagent and BSA as a standard.

The hydrogen peroxide content was estimated according to Harinasut et al. [7]. Samples were homogenized with 0.1% (w/v) TCA. Mixture was centrifuged at 12000 *g* for 15 min. To 0.5 mL of the supernatant, 0.5 mL of 10 mM phosphate buffer (pH 7.0) and 1 mL of 1 M potassium iodide (KI) were added. The mixture was incubated at 25°C for 15 min. The absorbance was measured at 390 nm. H_2O_2 content was calculated from a standard curve prepared in a similar way.

Lipid peroxidation was estimated from the amount of MDA formed in a reaction mixture [16]. Leaf tissues were homogenized in 0.1% (w/v) (TCA). The homogenate was centrifuged at 10000 *g* for 5 min. To 1 mL of the supernatant, 4 mL of 20% TCA containing 0.5% thiobarbituric acid was added. The mixture was incubated at 95°C in water bath for 30 min, and then quickly cooled on ice. The mixture was centrifuged at 10000 *g* for 15 min and the absorbance was measured at 532 nm. MDA levels were calculated from 1,1',3,3'-tetraethoxypropan standard curve.

All chemicals and reagents used in this experiment were purchased from Sigma Aldrich (Germany), Fluka (Germany) and Merck (Germany).

Statistical analysis. Experiments were conducted in complete randomized block design with 4 replications. Results indicated as mean values ± SD. Differences between control and treated seedlings were analyzed by Sigma Stat (3.02) with Tukey's Multiple Range Test ($p < 0.05$).

RESULTS

Salt stress ($p < 0.05$) significantly decreased fresh and dry weights of shoots in both cultivars at 150 mM NaCl. In non-saline conditions SA application had no significant effects on these parameters. Application of 250 μM SA for 75 mM NaCl-treated plants had no significant effects but improved the parameters in 150 mM NaCl-treated plants of both cultivars. Seedlings of both cultivars died at 750 μM SA + 150 mM NaCl, while application of 750 μM SA with 75 mM NaCl decreased the fresh and dry weights of shoots slightly in Azar2 but significantly in Chamran (table).

The activities of antioxidative enzymes, SOD and POD, significantly increased ($p < 0.05$) in both cultivars due to salt stress. Application of 75 mM NaCl increased SOD and POD activities in shoots by 140 and 91% in Azar2 and by 127 and 225% in Chamran,

Effects of salicylic acid (SA) on fresh and dry weights in two wheat cultivars under saline and non-saline conditions

Species	NaCl, mM	SA, mM	Fresh weight, g	Dry weight, g
Azar2	0	0	0.09 ± 1.36^{a}	0.003 ± 0.104^{ab}
	75	0	0.20 ± 1.16^{ab}	0.020 ± 0.105^{ab}
	150	0	0.09 ± 0.56^{d}	0.010 ± 0.056^{c}
	0	250	0.23 ± 1.23^{ab}	0.021 ± 0.089^{ab}
	75	250	0.23 ± 0.94^{bc}	0.020 ± 0.075^{ab}
	150	250	0.07 ± 0.79^{c}	0.030 ± 0.072^{b}
	0	750	0.21 ± 1.41^{a}	0.040 ± 0.120^{a}
	75	750	0.24 ± 1.01^{abc}	0.012 ± 0.078^{ab}
Chamran	0	0	0.18 ± 1.07^{a}	0.015 ± 0.080^{a}
	75	0	0.20 ± 0.95^{ab}	0.020 ± 0.08^{a}
	150	0	0.06 ± 0.37^{d}	0.005 ± 0.035^{c}
	0	250	0.10 ± 0.76^{abc}	0.010 ± 0.063^{abc}
	75	250	0.13 ± 0.72^{bc}	0.010 ± 0.068^{ab}
	150	250	0.18 ± 0.53^{c}	0.020 ± 0.054^{ab}
	0	750	0.14 ± 1.09^{a}	0.015 ± 0.085^{a}
	75	750	0.05 ± 0.57^{cd}	0.006 ± 0.053^{bc}

Each value represented as mean ± SE ($n = 4$); mean values followed by the same letter(s) are not significantly different ($p < 0.05$).

respectively. However, 150 mM NaCl caused a slightly increase in SOD and POD activities in both cultivars. Under the non-saline conditions, the application of 250 μM SA had no effect on the activities of these enzymes, but 750 μM SA resulted in maximum increase in activities of SOD and POD in both cultivars. The activity of SOD increased by 144 and 5% and the activity of POD increased by 38 and 68% in Azar2 and Chamran, respectively, compared to the control. In saline conditions application of 250 μM SA with 75 mM NaCl decreased the activities of SOD and POD in both cultivars, but there was significant increase in these enzyme activities of cultivars treated with 150 mM NaCl. Application of 750 μM SA in 75 mM NaCl-treated plants decreased significantly the activity of SOD in both cultivars, but POD activity was not affected in Azar2 and slightly decreased in Chamran (Fig. 1).

During NaCl treatment, CAT activity increased slightly in 150 mM NaCl-treated plants of Azar2 and 75 mM NaCl-treated plants of Chamran. There was small decrease in the activity of this enzyme in 150 mM NaCl for Chamran. Under non-saline conditions, the application of 250 μM SA had no significant effects on CAT activity in wheat cultivars, but 750 μM SA in seedlings of Azar2 increased the activity of CAT about four times, while seedlings of Chamran showed a slightly increase in CAT activity. Application of 250 μM SA with 75 mM NaCl-treated plants caused a high increase in this enzyme activity, the highest CAT activity was recorded in 250 μM SA + 75 mM NaCl for both cultivars. The increase in CAT activity induced by 250 μM SA in 150 mM NaCl-treated plants was not notable. Similarly, 750 μM SA application did not significantly affect CAT activity in cultivars treated with 75 mM NaCl (Fig. 2).

Salt stress significantly ($p < 0.05$) decreased the content of leaf soluble proteins in both cultivars at 150 mM NaCl. In non-saline conditions application of 250 μM SA had no effect on protein content in plants, but application of 750 μM SA caused a significant decrease in content of leaf soluble proteins in Azar2. In NaCl-treated plants of Azar2 and Chamran cultivars application of 250 and 750 μM SA decreased the protein content, while this reduction in Chamran was higher under 750 μM SA application ($p < 0.05$) (Fig. 3).

The contents of H_2O_2 and MDA in shoots of both cultivars increased significantly in 150 mM NaCl salinity. In non-saline conditions application of two SA levels had no effect on H_2O_2 and MDA contents in Azar2, but 750 μM SA increased significantly ($p < 0.05$) H_2O_2 content in Chamran. Under salinity conditions, application of 250 μM SA with reduced H_2O_2 content compared with the control in both cultivars, but MDA content was not affected significantly (Fig. 4).

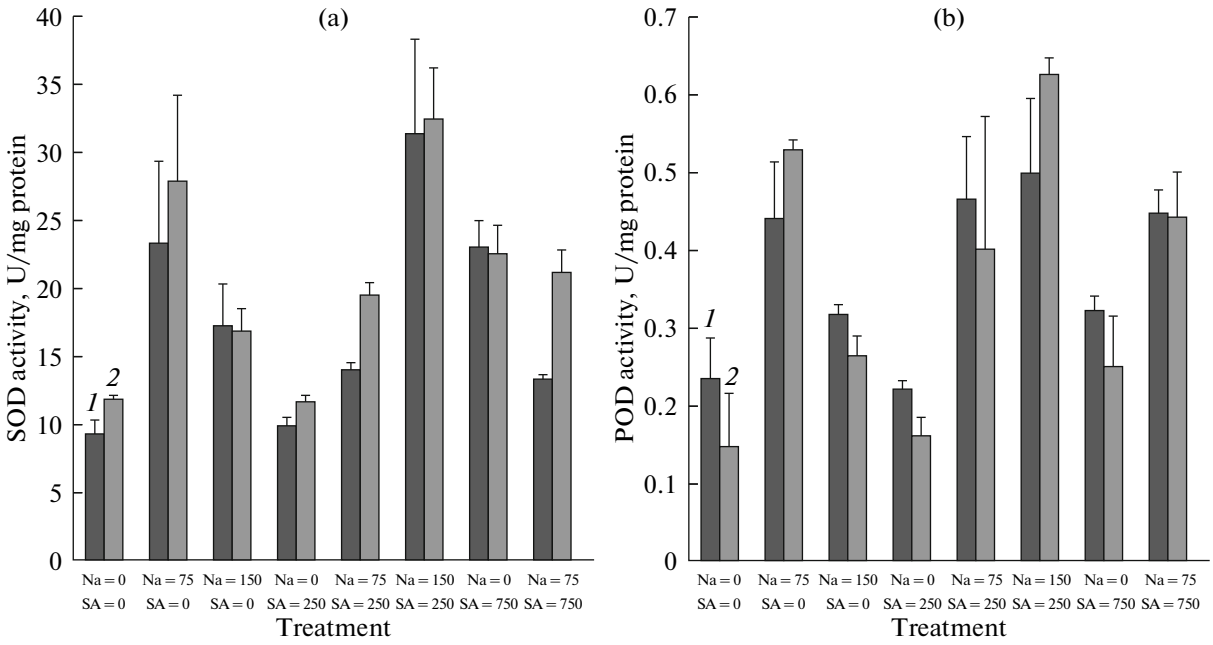

Fig. 1. Effects of salicylic acid on SOD (a) and POD (b) activities in two wheat cultivars under saline and non-saline conditions. *1*—Azar2, *2*—Chamran. Na—NaCl (mM) and SA—salicylic acid (μM) concentrations.

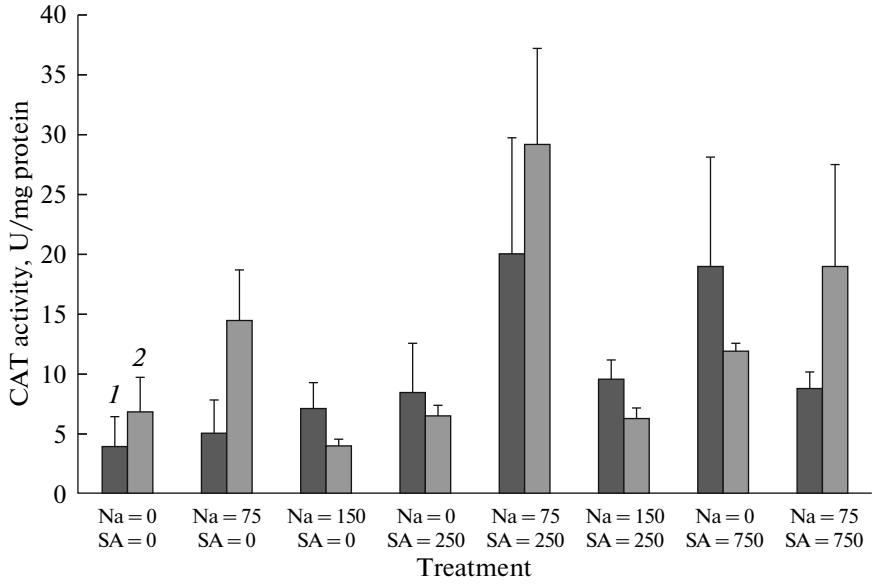

Fig. 2. Effects of salicylic acid on CAT activity in two wheat cultivars under saline and non saline conditions. *1*—Azar2, *2*—Chamran. Na—NaCl (mM) and SA—salicylic acid (μM) concentrations.

DISCUSSION

Two studied wheat cultivars showed sensitivity to 150 mM NaCl that had adverse effects on plant growth. These results are similar to those reported by Erdal et al. [17] who found that fresh and dry weights were reduced by salt stress in wheat plants. Changes in the metabolism of plants in response to salinity could be responsible for the diminished growth of plants under higher concentrations of NaCl [17]. Salt stress limits plant growth by adversely affecting various physiological and biochemical processes including photosynthesis, antioxidant capacity and ion homeostasis [18]. The ameliorative effects of SA application in improvement of detrimental effects of salinity in plants (that was seen in this study at 250 μM SA for both wheat cultivars), have been reported by several authors [17, 19, 20]. According to our results, even if the appli-

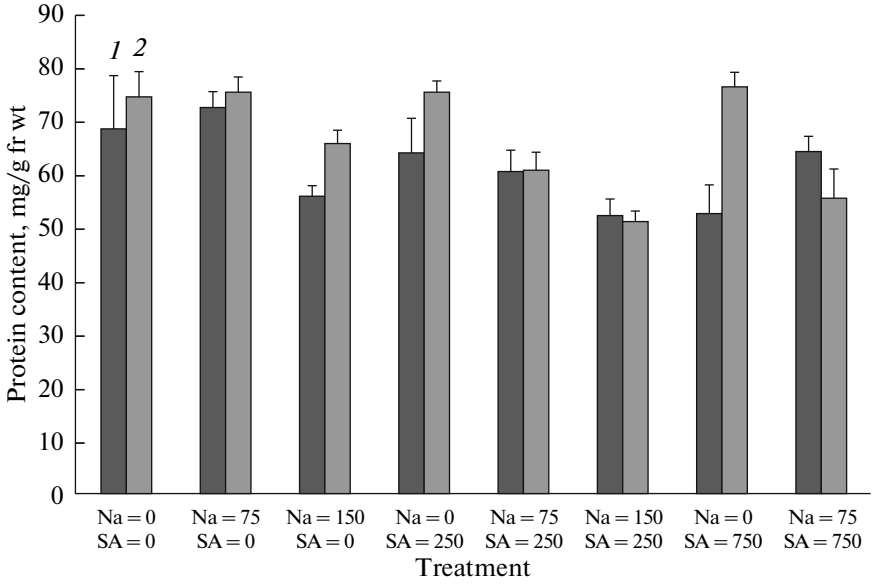

Fig. 3. Effects of salicylic acid on protein content in two wheat cultivars under saline and non saline conditions. *1*—Azar2, *2*—Chamran. Na—NaCl (mM) and SA—salicylic acid (μM) concentrations.

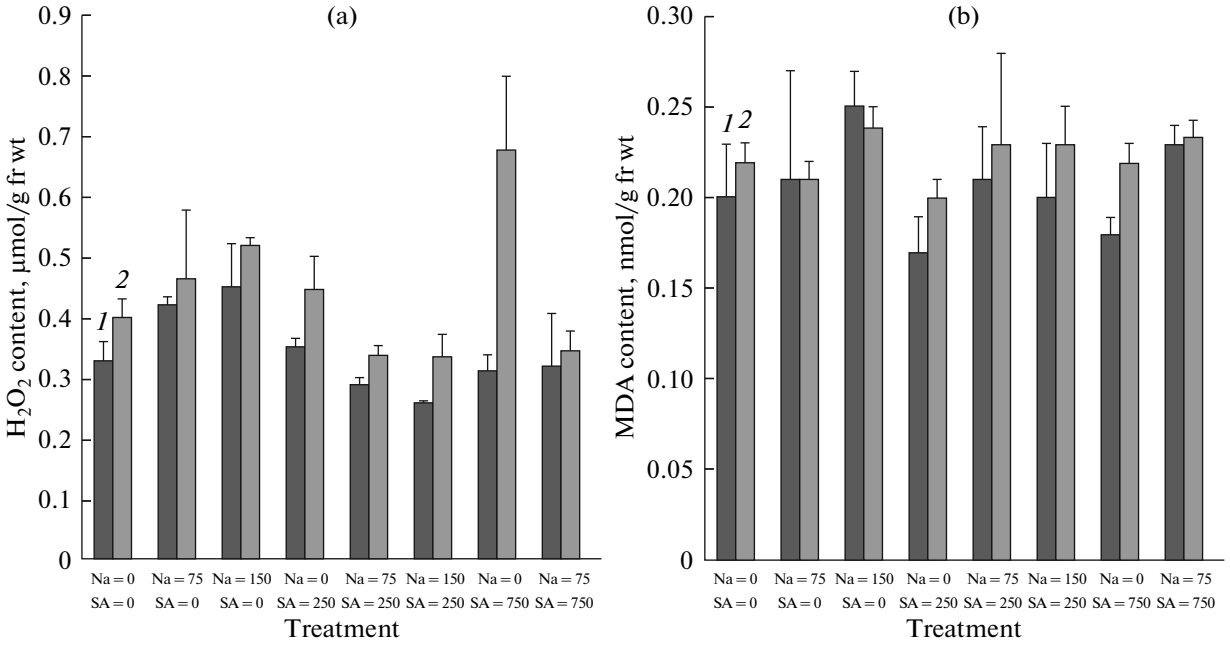

Fig. 4. Effects of salicylic acid on H_2O_2 (a) and MDA (b) contents in two wheat cultivars under saline and non saline conditions. *1*—Azar2, *2*—Chamran. Na—NaCl (mM) and SA—salicylic acid (μM) concentrations.

cation of 750 μM SA had no apparently adverse effect in non-saline condition on wheat cultivars growth, but it was injurious in saline conditions especially in Chamran that caused more decrease in growth parameters compared to Azar2. It is obvious that the beneficial effects of SA application in different environmental stresses depend on the types of species or cultivars [21]. The roles of SA in moderate and severe abiotic stresses may be contradictory and the fit concentration of SA that results to improve the stress effects is different among plant species [21, 22].

The increases in the activities of antioxidant enzymes SOD and POD in both cultivars and the activity of enzyme CAT in Chamran were seen in dimly salt stressed plants without SA application in this study. The increase in the activity of POD enzyme was higher in Chamran. The increasing in the activities of antioxidant enzymes could be the indicator of

build-up of a protective mechanism to reduce oxidative damages induced by stresses [6–8]. It seems that, Chamran produces high amount of H_2O_2 compared to Azar2 that effectively is removed by POD and CAT at low concentrations of NaCl. According to our results, wheat cultivars could not increase effectively their antioxidant enzymes activities in order to scavenging free radicals of oxygen in extreme salt stress, moreover a small decrease in CAT activity in Chamran was seen. In addition, application of low concentration of SA in these plants could result in increasing antioxidant enzymes activities. The increase in CAT activity by salt stress is a phenomenon that occurs in many kinds of plant species. In some cases, however, a decrease in CAT activity after NaCl treatment was observed in pea and rye [23], indicated that the responses may be different according to the intensity of the stress, plant part, plant species or cultivar, time assayed after stress treatment and induction of new isozyme(s) [17]. Several authors have reported the ameliorative effects of SA in salinity on antioxidant system of different plant species such as wheat [22, 24]. Increased SA-induced SOD activity causes a transient increase in H_2O_2 content, which in turn induces antioxidative enzymes (POD and CAT) leading to decrease in ROS on long term basis [25]. In the present study CAT and specially POD activities enhanced by SA application. Concurrently, SA stimulates the hydrolysis of soluble proteins, providing a pool of compatible osmolytes, which is important in osmotic adjustment in the presence of Na^+ [18, 24]. The results obtained in this study for the protein contents of wheat cultivars with application of SA in saline conditions are comparable to this view.

The contents of H_2O_2 and MDA of wheat cultivars increased at the higher concentration of NaCl. Salt stress induces water deficit and increases ionic and osmotic effects leading to oxidative stress and formation of ROS [26]. This increased ROS levels in plants can cause oxidative damage to biomolecules such as lipids, thus increasing the MDA content as the decomposition product of polyunsaturated fatty acids of membranes [27]. When applied exogenously at suitable concentrations, SA was found to alleviate the oxidative stress generated by salt stress [20].

In this study application of low concentration of SA in non-saline condition had not notable effects on wheat cultivars, but application of high concentration of SA resulted in substantial changes of studied parameters. As a response to high SA concentration in Azar2, activities of antioxidant enzymes were significantly increased and protein content was decreased. In Chamran the increase in SOD activity was very small compared to Azar2 and CAT activity decreased slightly. In addition, protein content of Chamran was not affected by SA, while H_2O_2 content significantly increased. These results suggest that the high concentration of SA (750 μM) is potent to cause stress conditions in wheat cultivars and Chamran was more susceptible than Azar2. The adverse effect of this level of SA in wheat cultivars was more obvious in saline conditions.

It has been proposed that SA have a dual role in plants. Firstly, SA is necessary for the induction of antioxidant defenses and maintaining the redox state of the cells. Thus, SA has been shown to be essential for the plant protection against the oxidative stress. Secondly, an excessive SA accumulation can induce a programmed cell death pathway, leading to hypersensitive reaction in response to stress [22] that was obtained in this study for wheat cultivars by application of 750 μM SA with 150 mM NaCl. Borsani et al. [22] reported that SA increased the oxidative damage generated by NaCl and osmotic stress in Arabidopsis SA-deficient transgenic line expressing a salicylate hydrolase gene.

In conclusion, this study showed that two wheat cultivars can tolerate to low degrees of salt stress via induction of antioxidative enzymes. In high salinity, application of SA in appropriative concentration ameliorates the adverse effects of salt stress in both cultivars, but high level of SA potentiated salt stress in wheat cultivars and Chamran is more susceptible than Azar2 in this view.

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