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Full Length Research Paper

Effects of salicylic acid and nitric oxide on antioxidant capacity and proline accumulation in *Glycine max* L. treated with NaCl salinity

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The influence of salicylic acid (SA), sodium nitroprusside (SNP) and their reciprocal effects (SA+SNP) on certain physiological parameters in soybean seedlings grown in saline and non-saline conditions was investigated. The changes of leaf area, shoot fresh, dry weights and content of photosynthetic pigments showed that the addition of 100 µM SA and/or 100 µM SNP markedly declined the oxidative damage to soybean plants treated with NaCl salinity. The results proved that the interaction of salicylic acid with nitric oxide donor significantly enhanced the activities of catalase (CAT), ascorbate peroxidase (APOX) and guaiacol peroxidase (GPOX). Also, they decreased the amounts of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) in soybean leaves under sodium chloride toxicity. In order to reduce the oxidative damage caused by NaCl stress, the protective role of SA+SNP was often better than that of SA and SNP alone. As well, it was observed that the accumulation of proline was apparently accelerated by these substances under salt stress and that the interaction of salicylic acid with nitric oxide has synergistic effects in decreasing the deleterious effects induced by NaCl salinity.

Key words: Salicylic acid, nitric oxide, NaCl salinity, soybean.

INTRODUCTION

Salt stress is an important environmental stress that constrains both ionic toxicity and osmotic stress to plants, leading to nutrition disorder and oxidative stress. Salinity especially sodium chloride stress often leads to increased production of reactive oxygen species (ROS) in plants. It has proposed that plants control the level of ROS with developing an antioxidant defense system comprising enzymes such as the superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPOX) and ascorbate peroxidase (APOX) which are responsible for scavenging accumulated ROS (Shi et al. 2007). The regulation of these antioxidant constituents by exogenous substances might mediate the plant tolerance to salt stress. The survival of plants under such a stressful condition depends on the plant's ability to perceive the stimulus, generate and transmit the signals and to initiate various physiological and biochemical changes (Hossain

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Abbreviations: SA, salicylic acid; **NO,** nitric oxide; **SNP,** sodium nitroprusside; **FW,** fresh weight; **MDA,** malondialdehyde; **AOE,** antioxidant enzymes; **CAT,** catalase; **APOX,** ascorbate peroxidase; **GPOX,** guaiacol peroxidase; **AsA,** ascorbic acid; **NaCl,** sodium chloride; **ROS,** reactive oxygen species; **Chl,** chlorophyll; **TCA,** trichloroacetic acid; **EDTA,** ethylene diamine tetra acetic acid ; **PMSF,** phenyl methyl sulphonyl fluoride ; **PVP,** poly vinyl pyrrolidine.

and Fujita, 2009). The molecules such as salicylic acid and nitric oxide have been suggested as signal transducers or messengers. These substances have obtained particular attention because of inducing a protective effect on plants under a biotic stresses.

Salicylic acid (SA, 2-hydroxybenzoic acid) is considered as a hormone-like endogenous regulator, which influences a range of diverse processes in plants, including seed germination, ion uptake and transport, membrane permeability and photosynthesis. Salicylic acid, acts as a potential non-enzymatic antioxidant and an important signal molecule for modifying plant responses to environmental stressors. Some earlier reports display that exogenous SA can ameliorate the impairing effects of drought stress in wheat (Waseem et al., 2006) and salt stress in maize and wheat (Arfan et al., 2007).

Nitric oxide (NO) is a lipophilic gas with a diffusion coefficient close to that of O_2 in aqueous solution that plays important role in diverse physiological processes. It has been reported that this molecule can regulate the response of numerous plants to a variety of stressors, such as drought (Garcia-Mata and Lamattina, 2007) and salinity (Zhang et al., 2006). Nitric oxide promotes normal growth and development of plants at lower concentrations (Beligni and Lamattina, 2001) that can be generated non-enzymatically, by chemical breakdown of NO donor molecules, such as sodium nitroprusside (SNP), *S*- nitroso-*N*- acetylpenicillamine, *S*-nitrosoglutathione (GSNO) and 3-morpholinosydnomine. Exogenous application of NO confers tolerance to various abiotic stresses in plants by enhancing both enzymatic and non-enzymatic antioxidant defense system (Xu et al., 2010).

Recently, it was established that NO elevates the content of salicylic acid known as a component of signaling pathways during biotic stresses (Klessig et al., 2000) and salicylic acid may in turn stimulate synthesis of NO in *Arabidopsis thaliana*, acting via enzyme with NO synthesizing activity (Zottini et al., 2007). In this paper, we studied the reciprocal effects of SA and SNP (as NO donor) under NaCl salinity on soybean seedlings. The results showed that SA and NO act synergistically to reduce the damaging effects of salt stress.

MATERIALS AND METHODS

Plant materials and plant treatments

Since soybean is an important agricultural crop and sensitive to salt stress that affects in terms of growth and yield, thus we chose it. Seeds of *Glycine max* L. cv. Union × Elf (called L17) were provided from the institute of sapling and seed in Tehran. We sterilized a lot of uniform seeds with sodium hypochlorite solution (5%) for five minutes and washed thoroughly with distilled water before using. Sterilized *G.max* seeds were placed in Petri dishes containing water between two filter papers, and kept at 25°C in an incubator. After germination, 7-day-old seedlings were cultivated in a hydroponics system in the growth chamber (14-h light period, day/night temperatures of 25/20°C respectively and 70% relative humidity). Salicylic acid (0 and 100 µM) was used as pre-treatment during the second week through 50% Hoagland's solutions before the application of NaCl salinity and NO donor. The different concentrations of NaCl (0, 50 mM, 100 mM) without or with SNP (0 and 100 µM) during the third week were added on the surface of the complete Hoagland solutions. SNP (sodium nitroprusside, Merck-Germany) was used as donor of NO. The Hoagland solutions related to each treatment were renewed once a day.

After one week pre-treatment with salicylic acid and then one week treatment with NaCl and SNP, the samples of leaf were collected, washed and used immediately to examine leaf area, shoot fresh and dry weights, the content of photosynthetic pigments and malondialdehyde (MDA), accumulation of proline and the activities of CAT, APOX and GPOX in soybean plants. Leaf area was measured with leaf area meter AM 200 (ADC Bioscientic Ltd, England).

Chlorophylls and carotenoids content

The photosynthetic pigments were extracted from 0.1 g leaf fresh weight by 80% acetone. The content of chlorophylls and carotenoids (xanthophylls and carotenes) were determined according to the procedure described by Lichtenthaler (1987).

$$\text{Chlorophyll.a} = C_a\,(\mu g.ml^{-1}) = 12.25\,A_{663.2} - 2.79\,A_{646.8}$$

$$\text{Chlorophyll.b} = C_b\,(\mu g.ml^{-1}) = 21.50\,A_{646.8} - 5.10\,A_{663.2}$$

$$\text{Carotenoid} = C_{x+c}\,(\mu g.ml^{-1}) = (1000A_{470} - 1.8C_a - 85.02C_b)/198$$

MDA content

Lipid peroxidation in Leaf fresh tissue (0.1 g) was determined by measuring malondialdehyde (Heath and Packer, 1968). The amount of MDA was calculated using the extinction coefficient ($\varepsilon = 155\ mM^{-1}\,cm^{-1}$) and expressed as $\mu mol.g^{-1}FW$.

Proline determination

Proline was extracted and determined by the method of Bates et al. (1973). Proline content was calculated with standard curve at 520 nm and expressed as $\mu mol.g^{-1}FW$.

H_2O_2 content

For determination of H_2O_2 concentration, leaf fresh tissue (0.1 g) was extracted with 3 ml TCA (0.1 %, w/v) in an ice bath and centrifuged at 12,000×*g* for 15 min (Velikova et al., 2000). The content of H_2O_2 was expressed as $\mu mol.g^{-1}FW$ using the extinction coefficient ($\varepsilon = 0.28\ mM^{-1}\,cm^{-1}$) at 390 nm.

Enzyme extraction and assay

Leaf fresh samples (500 mg) were ground in 5 ml of 50 mM phosphate buffer (pH 7.5) containing 1 mM EDTA, 1 mM PMSF and

Table 1. Effects of SA, NO donor SNP and SA+NO supply on leaf area, shoot fresh weight and shoot dry weight in soybean plants under saline and non-saline conditions.

Treatment			Leaf area plant^{-1} (cm^2)	Shoot fresh weight (g)	Shoot dry weight (g)
NaCl (mM)	SA (µM)	SNP (µM)			
0	0	0	134.92 ± 9.89	2.491 ± 0.134	0.33 ± 0.022
		100	151.82 ± 14.76	2.867 ± 0.232 *	0.352 ± 0.025
	100	0	153.69 ± 3.50 *	2.938 ± 0.090 *	0.350 ± 0.022
		100	132.44 ± 12.19	2.651 ± 0.217	0.326 ± 0.028
50	0	0	89.52 ± 9.74 *	1.961 ± 0.176 *	0.264 ± 0.028 *
		100	96.59 ± 10.27	2.184 ± 0.146	0.311 ± 0.025
	100	0	116.44 ± 8.90 *	2.693 ± 0.151 *	0.323 ± 0.020 *
		100	115.00 ± 13.72 *	2.642 ± 0.212 *	0.326 ± 0.028 *
100	0	0	59.23 ± 7.53 *	1.610 ± 0.116 *	0.211 ± 0.015 *
		100	78.72 ± 6.47 *	2.248 ± 0.145 *	0.267 ± 0.024 *
	100	0	64.16 ± 9.46	1.830 ± 0.150	0.250 ± 0.016 *
		100	85.05 ± 9.98 *	2.282 ± 0.148 *	0.305 ± 0.024 *

Mean ± SE was calculated for five replicates. The significance of difference between treatments was determined by Duncan's test. Values with the asterisk are significantly different at P< 0.05. The asterisks indicate a statistical difference at p<0.05.

1% PVP using pre-chilled mortar and pestle. Then, the extract was centrifuged at 4°C at 15,000×*g* for 30 min. The supernatant was used for measurements of enzyme activity and the activities of enzymes expressed as $\mu mol^{-1}.\min^{-1}.mg^{-1}$ protein. Protein content was determined using bovine serum albumin as standard (Bradford, 1976).

CAT (EC 1.11.1.6)

Catalase activity was assayed by monitoring the decrease in the absorbance of H_2O_2 using the method of Cakmak and Marschner (1992). Activity was calculated using an extinction coefficient ($\varepsilon = 39.4\ mM^{-1}cm^{-1}$) at 240 nm.

APOX (EC 1.11.1.11)

Ascorbate peroxidase activity of was measured by monitoring the oxidation of ascorbic acid (Nakano and Asada, 1981). Activity was calculated using an extinction coefficient ($\varepsilon = 2.8\ mM^{-1}cm^{-1}$) at 290 nm.

GPOX (EC 1.11.1.7)

Guaiacol peroxidase activity was determined using the guaiacol test (Zhang et al., 2005). The formed tetraguaiacol has maximum absorption at 470 nm. Activity was calculated using an extinction coefficient ($\varepsilon = 26.6\ mM^{-1}cm^{-1}$).

Statistical analysis

All data were subjected to analysis of variances according to the model for a completely randomized design using a SPSS program. The differences among mean of treatments evaluated by Duncan's multiple range tests at the 0.05 probability level. The asterisks indicate a statistical difference at p<0.05.

RESULTS

Growth

As it is shown in Table 1, the application of two levels of NaCl (50 mM and 100 mM as a low and high salinity) to *G. max* adversely decline leaf area, fresh and dry weights of shoot. In the absent of NaCl salinity, SA significantly increased leaf area and shoot fresh weight, but had no significant effect on shoot dry weight. Treatment with SNP clearly increased shoot fresh weight, while slightly enhanced leaf area and shoot dry weight of soybean plants under non-saline conditions. Also, the interaction of SA and donor of NO (SA+SNP) had no significant effect on leaf area, shoot fresh weight and shoot dry weight in the absent of NaCl salinity. Under low salinity, the measured growth parameters were markedly increased by application of SA and the combination of SA with NO donor through Hoagland solution, while SNP was not effective alone. Under high salinity, exogenous application of NO donor and SA+SNP increased leaf area, fresh and dry weights of shoot meaningfully, while SA only affected shoot dry weight.

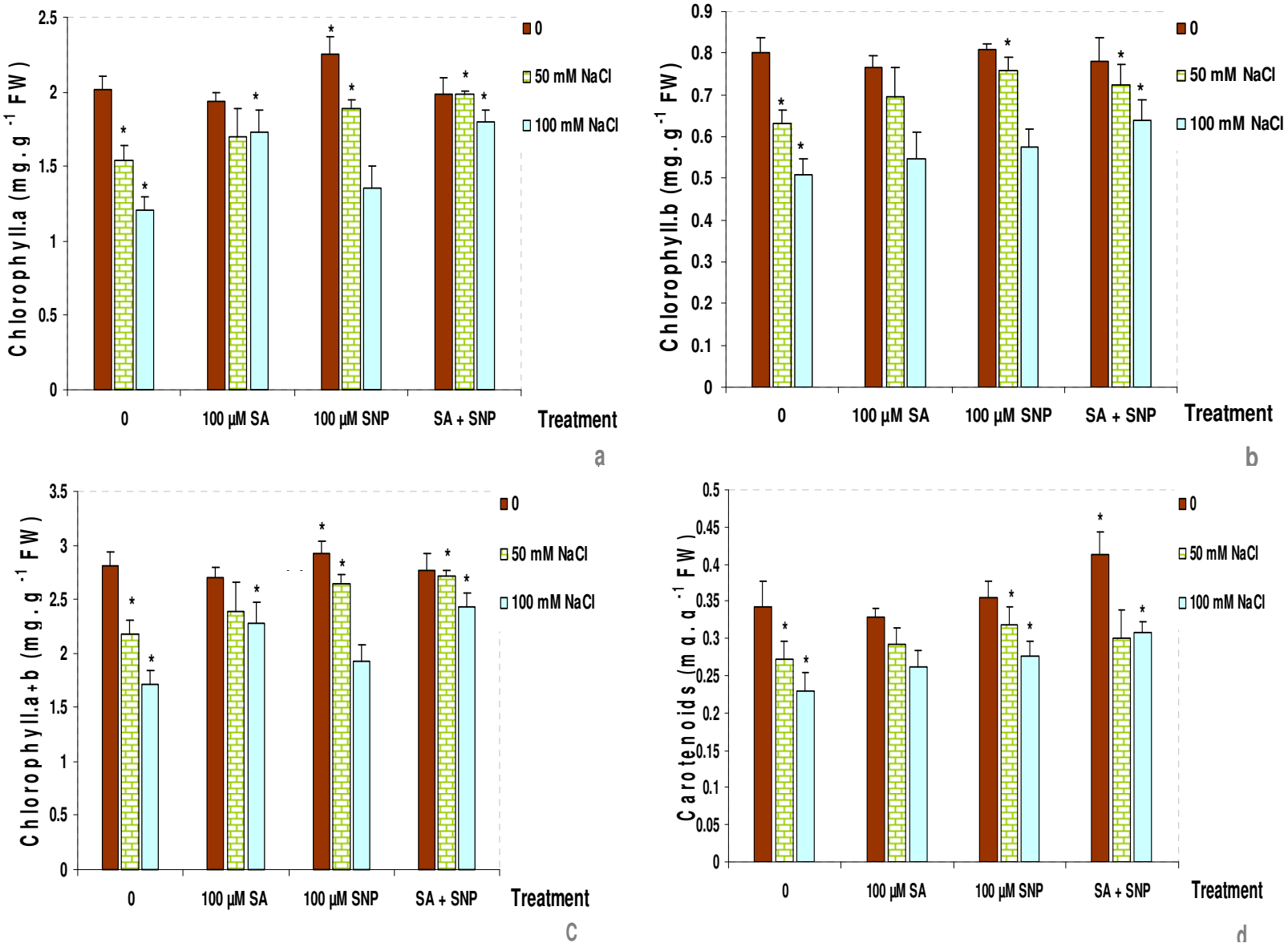

Figure 1. Effects of application of SA, SNP and SA+SNP on the amount of chlorophyll.a (1a), chlorophyll.b (1b), chlorophyll.a+b (1c) and carotenoids (1d) in soybean leaves under saline and non-saline conditions. Results are shown as Mean ± SE ($p<0.05$), obtained from five replicates. The asterisks indicate a statistical difference at $p<0.05$.

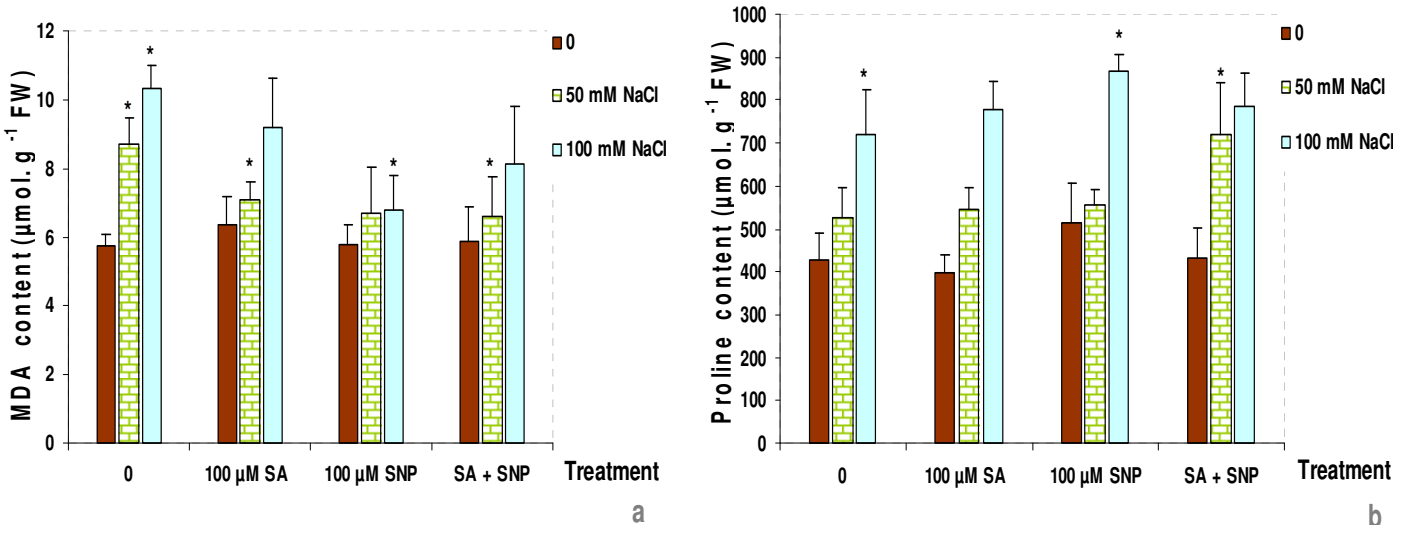

Figure 2. Effects of application of SA, NO donor and SA+SNP on malondialdehyde (MDA) content (a) and proline content (b) in leaves of soybean plant under salinity stress and non-saline control. Results are shown as Mean ± SE ($p<0.05$), obtained from four replicates.

Chlorophylls and carotenoids content

Due to the role of chlorophylls and carotenoids in the photosynthesis process, we measured the content of these pigments in *G. max* leaves under different treatments. As shown in Figure 1(a-d) when NaCl concentration increases, levels of photosynthetic pigments reduce significantly. Under low salinity, the amount of chlorophylls and carotenoids were markedly increased by NO donor SNP. In this condition, SA+SNP substantially enhanced concentration of chlorophylls, while the content of carotenoids was not affected. In the high salinity, the application of SA enhanced the content of Chl.a and Chl.a+b significantly, while the application of NO donor was only increased concentration of carotenoids. Also, the combination of SA with SNP clearly enhanced concentration of chlorophylls and carotenoids.

MDA content

Malondialdehyde is an indicator of lipid peroxidation that links to peroxidation of polyunsaturated fatty acids in the biological membranes and results in releasing free radicals. As shown in Figure 2a, the content of MDA significantly increased by 50 and 100 mM NaCl salinity. MDA content was declined in *G. max* treated with SA and SA+SNP in the presence of 50 mM NaCl and by application NO donor under 100 mM NaCl. In non-saline conditions, treatment with SA, SNP and SA+SNP slightly enhanced malondialdehyde content.

Proline concentration

Proline concentration at 50 mM and 100 mM NaCl enhanced 23 and 68% as compared with the control, respectively. As shown in Figure 2b, a significant increase in free proline accumulation was observed in response to 100 mM NaCl salinity, while increase in the content of proline under 50 mM NaCl was not significant. Our data showed that the amount of proline was strikingly enhanced by SA+SNP under low salinity and by NO under high salinity.

H_2O_2 content

Hydrogen peroxide content was increased in soybean plants which were treated with salinity, especially in high consecration of NaCl salinity. Our data in Figure 3a showed that in *G. max* treated with 100 mM NaCl, the application of SA, NO donor and SA+SNP could significantly decline concentration of hydrogen peroxide, while on exposure to 50 mM NaCl, these substances had

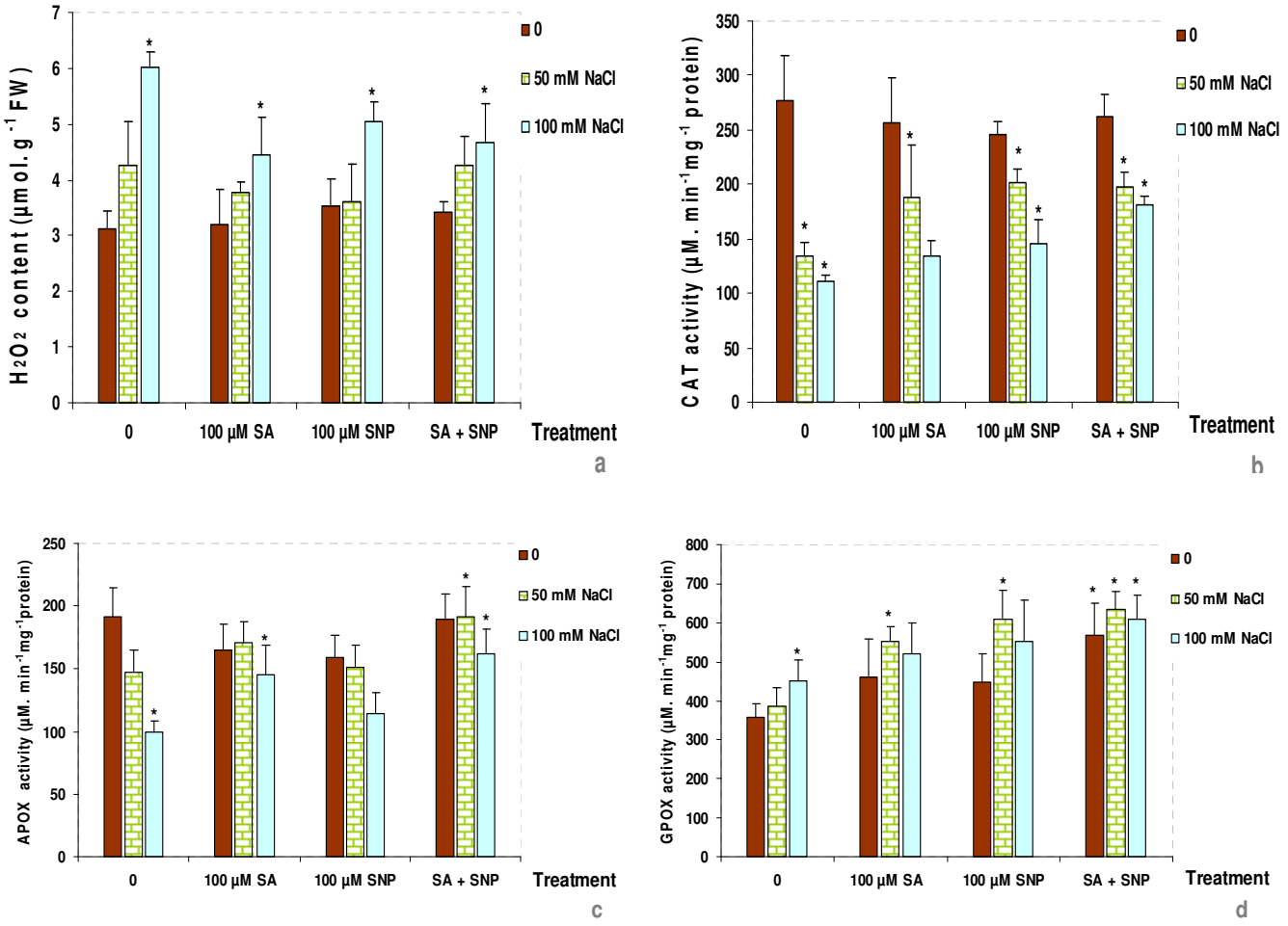

Figure 3. Effects of application of SA, SNP and SA+SNP on H_2O_2 content (a), catalase activity (b), ascorbate peroxidase activity (c) and guaiacol peroxidase activity (d) in leaves of soybean under non-saline and saline conditions. Results are shown as Mean ± SE ($p<0.05$), obtained from five replicates.

no significant role in reduction of H_2O_2 content. Based on results shown in Figure 3a, treatment of soybean seedlings with SA, SNP and the combination of SA with NO donor slightly increased the content of hydrogen peroxide in the absence of salt stress.

Antioxidant enzymes

Results in Figure 3b demonstrated that salt stress, particularly high salinity significantly decreased the activity of catalase, while exogenous application of NO donor and SA+SNP during 50 mM NaCl and treatment with SA, donor of NO and SA+SNP exposure to 100 mM NaCl markedly increased the activity of this enzyme. As shown in Figure 3(c, d), the activity of APOX enzyme in response to 100 mM NaCl salinity was considerably declined but the activity of GPOX increased. In the presence of 50 mM NaCl salinity, the activity of APOX by interaction of SA with SNP was significantly enhanced, while treatment with SA, NO donor and SA+SNP could clearly increase GPOX activity. Exposure to 100 mM NaCl, exogenous SA increased the activity of APOX enzyme and treatment with SA+SNP significantly enhanced activities of APOX and GOPX. In the non-saline conditions, exogenously applied SA, SNP and their interaction (SA+SNP) had no significant effect on activity of APOX but treatment with SA+SNP could raise GPOX activity significantly. Our results showed that the significant increase in the function of antioxidant enzymes caused salt tolerance in soybean exposed to NaCl salinity.

DISCUSSION

Our findings indicated that salicylic acid and nitric oxide act as plant growth regulators that can separately or together counteract NaCl-induced oxidative stresses. The results showed that under NaCl salinity, the effect of

combination of SA with NO in increasing leaf area, shoot fresh and dry weights was more than the effect of SA or NO donor alone. These results are in agreement with recent researches. It has been shown that salicylic acid reduces detrimental function of salinity and water deficient on growth of weath seedlings (Shakirova et al. 2003), Also exogenous NO increases dry weight of maize seedlings under salinity conditions (Zhang et al., 2006).

Measurement of photosynthetic pigments under salinity conditions on *G. max* explains that NaCl salinity has the negative effects on these pigments and treatment with SA or NO and especially the combination of SA with NO decreases damages induced by salt stress. Our results showed that salinity reduced the chlorophylls and carotenoids content in leaves of soybean plants. Furthermore, the effect of severe salinity is higher than low salinity. It has been observed that exogenous application of SA, NO and their reciprocal effects increase the content of photosynthetic pigments under NaCl salinity. It has been suggested that exogenous SA and NO lead to the promotion of protective reactions to the photosynthetic pigments and induce a pre-adaptive response to salt stress (El-Tayeb, 2005; Singh et al., 2008), which confirm our results.

Peroxidation of lipids was increased significantly in the *G. max* plants which were treated with salt stress resulting of sodium chloride, especially in high salinity. Exogenous application of SA, SNP and SA+SNP reduced NaCl-induced increase in the content of MDA. Nitric oxide decreases accumulation of hydroxyl in salt treated wheat leaves by eliminating O_2^- and H_2O_2 (Tan et al., 2008) and also, SA decreases the content of MDA by inhibiting production of hydroxyl radical (Gunes et al., 2007).

Under NaCl salinity proline accumulation was observed in our results. Exposure to 100 mM NaCl salinity, SA, NO and the interaction of SA with NO could enhance the content of this amino acid to confer resistance to salinity in soybean seedlings. It is possible that salicylic acid and/or nitric oxide can affect proline concentration through genes involved in the proline biosynthesis. Proline is able to scavenge hydroxyl radical and stabilize the structure and function of macromolecules such as DNA, protein and membranes via interaction with these macromolecules. It has been reported SA and NO through induction of ABA-mediated proline production can decrease the deleterious effects of osmotic stress in wheat (Sakhabutdinova et al., 2003; Tan et al., 2008). In salt-stressed soybean plants, the protective role of SA and NO may be related to its regulation effects on proline levels.

Electron transport processes such as photosynthesis and respiration generate basal levels of H_2O_2, which increase in response to stress. We observed that treatment with NaCl enhanced the content of H_2O_2 and the activity of GPOX, while reduced the activities of CAT and APOX. In Cd-exposed rice plants, pretreatment with SA increased the activity of GPOX (Guo et al., 2009). Increase of H_2O_2 concentration under salt stress is related to reduce of the activities of CAT and APOX. The activity of CAT was enhanced by SA, SNP and SA+SNP in the presence of NaCl salinity, which was declined in the absence of salt stress. The accumulation of H_2O_2 in SA-pretreated soybean plants in the absence of salt stress also might refer to the lower scavenging ability of H_2O_2 by the stable CAT activity and reduction in activities of APOX. It has been suggested that stimulation of antioxidants might be achieved by SA-induced protein synthesis (Kovacik et al., 2009). Treatment with exterior NO significantly raised the activity of APOX in cucumber leaves under salt stress (Fan et al., 2008). It was suggested when NO/ O_2^- ratio is in favor of NO, superoxide anion (O_2^-) combines with NO and produces Peroxynitrite ($ONOO^-$), thus there is no superoxide anion to convert to H_2O_2; finally the amount of H_2O_2 will decrease in the presence of exogenous NO (Delledonne et al., 2001). Peroxynitrite has been shown to combine with H_2O_2 to produce nitrite ion and oxygen (Beligni and Lamattina, 2001). Therefore, the abilities of nitric oxide in scavenging O_2^- in salt-treated soybean plants can benefit in stabilizing the CAT activities, and it appears that the same mechanism is operating in *G. max*. The application of SA and NO increase the activity of antioxidant enzymes and can ameliorate the toxic effects generated from NaCl.

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