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BRIEF COMMUNICATION

Physiological effects of exogenous nitric oxide on *Brassica juncea* seedlings under NaCl stress

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Abstract

The study was conducted to investigate the physiological effects of exogenous NO on potherb mustard (*Brassica juncea* Coss.) seedlings under salt stress. The plants were grown in Hogland nutrient solution for 15 d and treated with 150 mM NaCl, NO donor sodium nitropruside (SNP) and NO scavenger methylene blue (MB-1) for 4 d. The NaCl stress increased superoxide dismutase, peroxidase and ascorbate peroxidase activities and malondialdehyde (MDA) and free proline contents, and decreased soluble protein content. However, the application of exogenous NO limited the production of MDA and free proline, while markedly promoted SOD, POD and APX activity.

Additional key words: antioxidative enzymes, malondialdehyde, methylene blue, proline, sodium nitropruside.

Salinity adversely affects plant metabolism and causes modifications in growth, development, and gene expression (Amini *et al.* 2007). The effects of soil salinity on plants are due to a low osmotic potential of soil solution and specific effects of high Na^+ and Cl^- contents (Hasegawa and Bressan 2000). To avoid this adverse effects, plant evolves a great variety of adaptive mechanisms, such as osmotic adjustment and ion compartmentation (Nandwal *et al.* 2000). As a consequence of these primary effects, secondary stresses, such as oxidative damage due to production of reactive oxygen species (ROS), often occur (Sairam *et al.* 2002, Arora *et al.* 2008, Banu *et al.* 2009, Guo *et al.* 2009). Plants have different adaptive mechanisms to reduce oxidative damage resulting from salt stress through a cascade of antioxidants (Hichem *et al.* 2009) and antioxidant enzymes including superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), peroxidase (POD) and catalase (CAT), which play critical roles in neutralizing the free radicals (Allen and Tresini 2000).

Nitric oxide (NO) is a water and lipid soluble gaseous compound with high diffusion coefficient (Abat *et al.* 2008). NO can mediate plant responses to biotic and abiotic stresses including drought (Tian and Lei 2006, Zhang *et al.* 2009) and salinity (Sheokand *et al.* 2008, Xie *et al.* 2008). Although there is a large literature studying the function of NO and the relationship between NO and ROS in plants under salt stress, little has been reported on the time effect of the specific scavenger of NO, such as 2-(4-carboxyphenyl-4,4,5,5-tetramethylimid-azoline-1-oxyl-3-oxide (PTIO) or methylene blue (MB-1), and the reversed effect of them on different physiological parameters.

Potherb mustard (*Brassica juncea* Coss.) is a very popular and important leafy vegetables and its productivity is often limited by salinity. Therefore, the aim

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Abbreviations: APX - ascorbate peroxidase, MB-1 - methylene blue; MDA - malondialdehyde; POD - peroxidase; PTIO - 2-(4-carboxyphenyl-4,4,5,5-tetramethylimid-azoline-1-oxyl-3-oxide; SOD - superoxide dismutase; SNS - sodium nitropruside.
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of this study was to analyze the effects of salt stress on physiological parameters of potherb mustard seedlings and to elucidate protective mechanism of exogenous NO in alleviating salt injury.

Seeds of potherb mustard (*Brassica juncea* Coss.) were germinated and the 20-d-old seedling were transferred to a cultivation chamber and grown under relative humidity of 70 - 80 %, day/night temperature 25/15 ºC, and 16-h photoperiod with a photosynthetic photon flux density (PPFD) of 350 μmol m^{-2} s^{-1} (measured at the top of the plants with a *190 SB* quantum sensor, *LI-COR*, Lincoln, NE, USA). The plants were grown in the hole of foam-board suspending on the top of nutrient solution of pots (ϕ 20 cm), each foam-board containing eight plants. In the entire experiment, all the plants received a nutrient solution (pH 5.5) consisting of 6 mM KNO_3, 5 mM $CaCl_2$, 1 mM NaH_2PO_4, 2 mM $MgSO_4$, 0.025 mM H_3BO_3, 2 μM $MnCl_2$, 1 μM $ZnSO_4$, 0.1 μM $(NH_4)_6Na_7MoO_{24}$, 0.25 μM $CuSO_4$ and 10 μM Fe-EDDHA. The nutrient solution was renewed every 3 d and the *Vermiculite* partly rinsed with *Millipore*-filtered water in order to avoid nutrient accumulation. At day 15 after planting, several treatments were used: *1*) Hoagland solution (control), *2*) Hoagland solution containing 0.1 mM sodium nitropruside (SNP), *3*) Hoagland solution containing 5 μM MB-1, *4*) Hoagland solution containing 150 mM NaCl, *5*) Hoagland solution containing 0.1 mM SNP and 150 mM NaCl and *6*) Hoagland solution containing 0.1 mM SNP, 150 mM NaCl and 5 μM MB-1. Then, the second fully expanded leaf in each treatment was removed at the 4th day immediately for further measurements. The experiment was repeated four times under the same conditions.

RWC was estimated according to the method of Whetherley (1950). SOD activity was assayed by monitoring the inhibition of photochemical reduction of nitro-blue tetrazolium according to the method of Bayer and Fridovich (1987). POD activity was measured by following the change of absorption at 436 nm due to guaiacol oxidation (coefficient of absorbance of 6.39 mM^{-1} cm^{-1}) following Pütter (1974). Malondialdehyde (MDA) content was determined by the thiobarbituric acid reaction as described by Heath and Packer (1968). The free proline content in leaves was estimated according to the method of Bates *et al.* (1973), and sample was extracted in sulphosalicylic acid. Soluble protein content was determined following Bradford (1976) with a bovine serum albumin as a standard. All experiments were repeated three times, and statistical procedures were performed using the *PC-SAS* software package. Differences between means were determined using Duncan's multiple range test.

It was observed that RWC decreased markedly under NaCl treatment, and there was a decrease of 34 % at the fourth day after salt treatment (Table 1). Reductions in RWC under salinity stress may be attributed to decreased water uptake due to low substrate water potential, or to injury to the root system (Garg and Singla 2009). Pretreatment with 0.1 mM SNP protected against the decrease of RWC under salt stress. However, when experiment was performed in the presence of 5 μM MB-1, a specific scavenger of NO, RWC loss was similar to that found in NaCl-treated plants. Furthermore, without salt stress, the treatment of SNP and MB-1 alone did not have any effect on RWC. There are similar reports in alfalfa (Nandwal *et al.* 2000) and mungbean (Kabir *et al.* 2004) under salt stress conditions. However, the result was inconsistent with the observation by Sheokand *et al.* (2008), who found that NO had no significant effect on RWC under salt stress.

Table 1. Effects of exogenous NO on RWC [%], soluble protein [mg g^{-1}(f.m.)], MDA [mmol g^{-1}(f.m.)] and free proline [μg g^{-1}(f.m.)] contents in the leaves of potherb mustard seedlings under NaCl stress. Means of 4 replicates. Different letters indicate significant differences ($P < 0.05$) according to Duncan's multiple range tests. For treatments 1 - 6 see text.

Treatments	RWC	Protein	MDA	Proline
1-control	85.56a	21.31a	10.62c	270.31d
2-control+SNP	85.47a	19.86ab	9.13c	268.45d
3-control+MB-1	85.40a	18.52b	10.48c	264.17d
4-NaCl	56.41b	6.98d	20.25a	434.05a
5-NaCl+SNP	85.14a	10.81c	14.98b	304.27c
6-NaCl+SNP+MB-1	57.12b	7.88d	19.02a	408.34b

Salt stress led to a significant reduction in protein content, however, the addition of SNP could significantly increase the content of soluble protein compared with the treatment of NaCl alone. Moreover, MB-1 could distinctly reverse the effect of SNP (Table 1). Similar results were reported by Arora *et al.* (2008) in seedlings of *Zea mays* and Aghaleh *et al.* (2009) in *Salicornia persica* and *S. europaea*.

MDA has been frequently described as a suitable biomarker for lipid peroxidation (Bailly *et al.* 1996). In the present study, the content of MDA in the seedling leaves under salt stress increased sharply (Table 1). Similar results were observed by Sheokand *et al.* (2008) and Banu *et al.* (2009). However, application of exogenous NO caused the slight reduction of MDA content under control conditions and less increase in MDA content under salt stress (Table 1). The result was in accordance with the observations in Chinese cabbage (López-Carrión *et al.* 2008) and chickpea (Sheokand *et al.* 2008). The increased MDA content might be due to the membrane destruction resulting from ROS-induced oxidative damage (Zhang *et al.* 2007), and exogenous NO might be involved in ROS scavenging.

Proline is a compatible solute that accumulates in great quantities under osmotic stress and participates in osmo-regulation and osmo-protection (López-Carrión *et al.* 2008). A stable proline content was observed in the

SNP and MB-1 treatments and in the control (Table 1). However, the proline content enhanced significantly under salt stress and the addition of SNP partially decreased this enhancement. The protective effect of SNP was reversed by the addition of MB-1. The result was consistent with the observation by López-Carrón *et al.* (2008).

Abiotic stress conditions favour the accumulation of ROS and their metabolism is dependent on antioxidant enzymes such as SOD, CAT, POD and APX. In the present study, there were no significant differences among the NO treatments in SOD, APX, and POD activities. However, the activities showed a steep rise in salt treatments, indicating an induction of the defense system. The treatment of NaCl with SNP caused a further significant ($P < 0.05$) increase in SOD, POD and APX activities (Fig. 1). However, the effect of SNP was reversed entirely or partially by the addition of MB-1. For example, there was no difference in SOD and POD activities between the treatment of NaCl and NaCl with SNP and MB-1, suggesting that MB-1 entirely inhibited the effect of SNP on SOD and POD. Nevertheless, in APX, MB-1 did not entirely prevent the effect of SNP, because there was significant difference between the NaCl alone and the treatment of NaCl with SNP and MB-1. The results were not entirely consistent with other reports. For instance, Sheokand *et al.* (2008) found that NO was effective in scavenging H_2O_2 by increasing the activities of CAT, POX and APX under salt stress, while putrescine was more effective in scavenging superoxide radical, as it increased the SOD activity under salt stress. On the other hand, Ruan *et al.* (2002) observed that exogenous NO significantly enhanced the activities of SOD and CAT, but it did not have any obvious effect on POD activity under salt stress. It is interesting to note that NaCl increased POX activity in the salt-tolerant while decreased in the salt-sensitive wheat cultivar (Mutlu *et al.* 2009). As concerns the other NO scavengers, Qian *et al.* (2009) reported that another NO scavenger, PTIO, could abolish all the protective effect of NO against herbicide damage. Similarly, Noriega *et al.* (2007) found that PTIO could arrest NO-mediated protective effects against to Cd-induced oxidative damage in soybean leaves. In present experiments, endogenous proline content increased simultaneously with antioxidant enzyme activities in the salt-stressed plants, and exogenous proline or betaine increased POD activity (Hoque *et al.* 2007) and lignin-forming POD expression (Banu *et al.* 2009). However, under Cd stress, neither exogenous proline nor betaine increased POX activity (Islam *et al.* 2009). In fact, many contradictory results about antioxidant enzymes response to different stresses have emerged due to the fact that the enzyme responses depend on the plant species, the developmental stage, the organs, as well as on the duration and severity of the stress (Reddy *et al.* 2004).

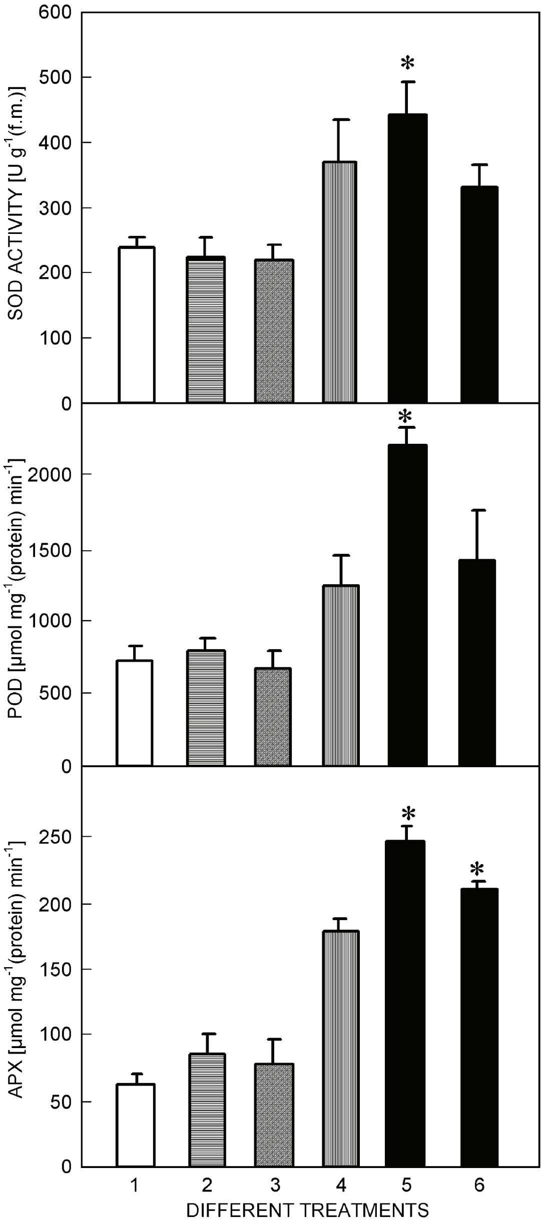

Fig. 1. Effects of exogenous NO on the activity of SOD, POD and APX in the leaves of potherb mustard seedlings under NaCl stress. All parameters were measured at the 4th day after application. Means ± SE, *n* = 4, * - values significantly different at $P < 0.05$ according to Duncan's multiple range tests. For different treatments see Table 1.

To conclude, our results suggested that exogenously supplied NO contributed significantly to the resistance of potherb mustard to NaCl and had an ameliorating effect on NaCl-induced oxidative stress. However, further research is required to determine the changes of these physiological parameters at the molecular level and the particular genes that are induced to bring such an effect.

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