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ORIGINAL ARTICLE

Comparison the effects of nitric oxide and spermidin pretreatment on alleviation of salt stress in chamomile plant (*Matricaria recutita* L.)

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Salt stress is an important environmental stress that produces reactive oxygen species in plants and causes oxidative injuries. In this investigation, salt stress reduced the shoot and root length, while increased the content of malondealdehyde, Hydrogen peroxide, and the activity of Ascorbate peroxidase andguaiacol peroxidase. Pretreatment of chamomile plants under salt stress with sodium nitroprussideand Spermidin caused enhancement of growth parameters and reduction of malondealdehyde and Hydrogen peroxide content. Pretreatment of plants with sodium nitroprusside remarkably increased Ascorbate peroxidase activity, while Spermidin pre-treatment significantly increased guaiacol peroxidase activity. Application of sodium nitroprusside or Spermidin with Methylene blue which is known to block cyclic guanosine monophosphate signaling pathway, reduced the protective effects of sodium nitroprussideand Spermidin in plants under salinity condition. The result of this study indicated that Methylene blue could partially and entirely abolish the protective effect of Nitric oxide on some physiological parameter. Methylene blue also has could reduce the alleviation effect of Spermidin on some of parameters in chamomile plant under salt stress, so with comparing the results of this study it seems that Spermidin probably acts through Nitric oxide pathway, but the use of2-4- carboxyphenyl- 4,4,5,5- tetramethyl-imidazoline-1-oxyl-3-oxide is better to prove.

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Key words: Antioxidant enzymes / Methylene blue / Polyamines / Salinity / Sodium nitroprusside

Excess amount of salt in the soil adversely affects plant growth and development. Processes such as seed germination, seedling growth, vegetative growth and flowering are adversely affected by high salt concentration, ultimately causing reduced economic yield and a quality of produce. High salt concentrations decrease the osmotic potential of soil solution creating a water

stress in plants. Secondly, they cause severe ion toxicity, since Na^+ is not readily sequestered into vacuoles. Finally, the interactions of salts with mineral nutrition may result in nutrient imbalances and deficiencies. The consequence of all these can ultimately lead to plant death because of growth arrest and molecular damage. Different plant species have developed different mechanisms to cope with these effects in response to salt stress (McCue and Hanson 1992). However, exposure of plants to salt stress can increase the production of reactive oxygen species (ROS). These species are highly reactive and can damage chlorophyll, proteins, lipids and nucleic acids (Foyer and Noctor, 2000). Plants process both enzymatic and non-enzymatic mechanisms are designated to minimize the concentration of ROS.

Nitricoxide (NO) is as mall and lipophilic gas and a bioactive molecule that play an important role in different physiological processes. There is increasing evidence showing that NO acts like a signal molecule in processes such as growth and development, respiratory metabolism, cell death, and ion leakage (Kopyra and Gwozdz 2004; Lamotto *et al.,* 2005). On the other hand, NO can also mediate plant growth regulators and ROS metabolism and increasingly evident have shown, which is involved in signal transduction and responses to biotic and abiotic stress such as drought, low and high temperatures, UV and ozone exposure, heavy metal, herbicides, cold, and salt stress (Neill and Desikan 2003; Del Rio *et al.,* 2004; Fan *et al.,* 2007).Tolerance to drought, salt and heat stress was enhanced in wheat (*Triticum aestivum*) and rice (*Oryza sativa*) seedlings when the plants were treated with NO donor, sodium nitroprusside (Mata and Lamattina 2001; Uchida *et al.,* 2002). In plant cells, the diamineputrescine (Put), triaminespermidine (Spd) and tetraminespermine (Spm) constitute the major PAs. They are known to be essential for growth and development (Tabor and Tabor 1984). The protective role of PA in plant stress reported in many studies (Zhao and Yang, 2008; Zhao *et al.*, 2008, Liu *et al.*, 2006).In previous researches it has been reported that the many effects of polyamines in alleviation of stresses may be related to production of NO and NO signaling pathway (Tun *et al.*, 2006). It is an interesting study of using Methylene blue (MB) to inhibit the NO signaling pathway. In organism, NO may act through activation of guanylatecyclase, which produces the second messenger cyclic GMP (cGMP), or through s-nitrosylation of redox-sensitive transcription factors or ion channels (Stamler, 1994). It has been reported that (MB) inhibits soluble guanylatecyclase and thereby the action of NO and cGMP (Keaney *et al*., 1994; Paciullo *et al*., 2010). There is another NO inhibitors or scavengers such as (2-4- carboxyphenyl- 4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (PTIO) but study on MB is lesser than PTIO. Based on the above observations, the objective of the present experiment was comparing the physiological mechanisms of exogenous NO and Spd with or without MB in increased chamomileplant tolerance to salinity stress.Comparing these responses can be useful in understanding the physiological and biochemical mechanisms of these compounds in plants which have to cope with salt stress.

MATERIALS AND METHODS

Chamomile (*Matricaria recutita L*.) seeds were sown in perlite and cocopite. After germination, seedlings were supplied with half strength of lang-Ashton nutrient solution three times a week. Sodium nitroprusside (SNP) was used as NO donor and methylene blue (MB) as inhibitor of NO

pathway. Two-month-old chamomileplants were obtained and watered separately with half strength Lang-Ashton solution with SNP (100µM), Spd (0.5mM), MB (100 µM), (MB + SNP) and (MB +Spd) for 10 days. Nutrient solution was used as control. After 10 days, all plants were divided in two groups one group was exposed to NaCl (200 mM) stress and other group was irrigated with water alone as control for 12 days.

Lipid peroxidation: For the measurement of lipid peroxidation in leaf rosettes, the thiobarbituric acid (TBA) test, which determines malondialdehyde (MDA) level, was applied (Heath and Packer, 1968).

H_2O_2 content: Hydrogen peroxide levels were determined according to Alexieva *et al.*, (2001).

Enzyme extraction and antioxidant enzyme activity: Leaf fresh samples (500 mg) were ground in 5 ml of 50mM phosphate buffer (pH 7.5) containing 1mM EDTA, 1mM PMSF and 1% PVP using pre-chilled mortar and pestle. Then, the extract was centrifuged at 4 ℃ at 15,000 g for 30 min. The supernatant was used for measurements of enzyme activity and the activities of enzymes expressed as Unit/mg protein^{-1} (Bradford, 1976). Ascorbateperoxidase activity was measured by monitoring the oxidation of ascorbic acid (Nakano and Asada, 1981) and GPX activity was determined using the method of Plewa *et al.* ,(1999).

Statistical analysis: Data are means± SE of three replicates. Statistic assays were carried out by one-way ANOVA using Duncan test to evaluate whether the means were significantly different, taking $p< 0.05$ as significant.

RESULTS

Growth parameters: Salt stress (NaCl) significantly decreased length of both shoot and root of *M. recutita* plants (Figure 1). Pretreatment of plants with SNP and Spm significantly enhanced the growth of chamomile plants under saline condition. However, pretreatment of plants with (SNP+ MB), and or (Spm +MB) had the same effects on growth parameters under salinity stress when compared with SNP or Spm pretreated plants. In the non-saline conditions, exogenously applied SNP, Spm had no significant effect on length of shoot and root.

MDA and H_2O_2 content: results in Figure 2 demonstrated that salt stress, significantly increased the content of MDA. Under salt stress lipid peroxidation decreased in plants which were pretreated with SNP and Spd when compared with non-pretreated plants. Application of SNP or Spd with MB as pretreatment mitigates the effects of SNP and Spm. The results showed that salt stress increased H_2O_2 content, whereas SNP and Spd treatment significantly decreased the amounts of H_2O_2 in leaf rosettes of *M. recutita*. Exogenous application of (MB + SNP) and (MB + Spd) during 200mM NaCl had no significant effect on H_2O_2 content.

APX and GPX activites: the effect of salt stress on GPX and APX in chamomile plant leaves, either with or without SNP and Spd pretreatment was assayed. As is shown in (Fig. 3) the activity of GPX (Fig. 3-A) and APX (Fig. 3-B) was higher in stressed plants than those of the control groups, which may be a reflection of the oxidative burst under salt stress and the key role of these enzymes in ROS detoxification under these conditions. SNP pretreatment had no significant effects on GPX activity, while pretreatment of plants with Spd significantly enhanced the activity of this enzyme under stress condition. As shown in Figure 3-B, the activity of APX enzyme was considerably increased

in response to salinity stress. Application of SNP pretreatment increased the activity of APX in salt stressed plants while the Spd pretreatment increased the APX activity in control plant and had no significant effect on activity of this enzyme in stress condition. Application of MB with SNP or Spd as pretreatments decreased the positive effects of these compounds on enzymes activity in saline conditions.

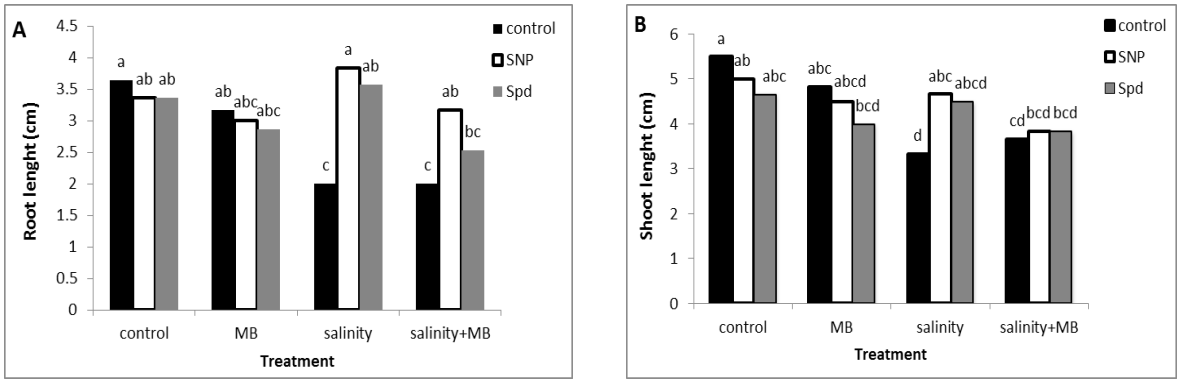

Figure 1. Effect of SNP, Spd and MB treatments on the length of shoot (A) and root (B) in chamomile plant leaves under control and salt stress condition. Data are means ± SE of three replicates. The significant of different between treatments was determined by one-way ANOVA taking $p<0.05$ as significant.

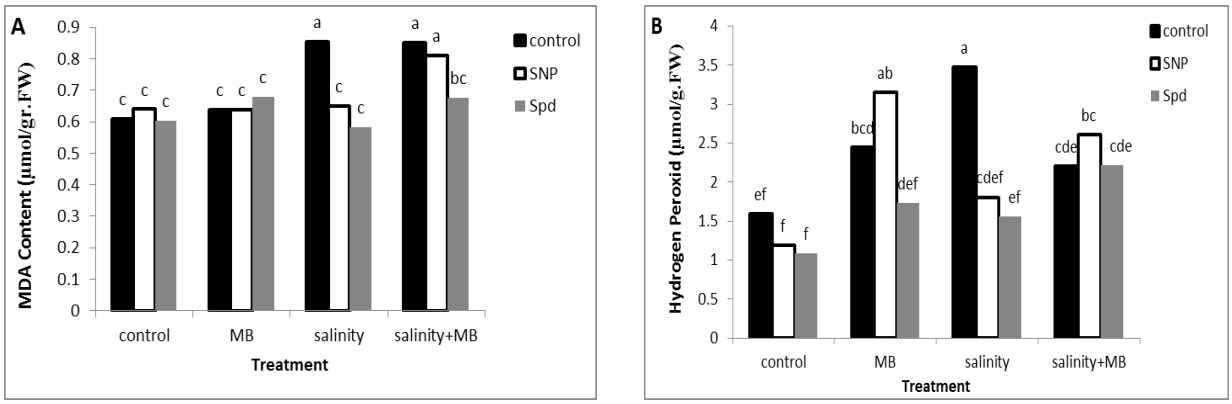

Figure 2. Effect of SNP, Spd and MB treatments on the contents of MDA (A) and H_2O_2 (B) in chamomile plant leaves under control and salt stress condition. Data are means± SE of three replicates. The significant of different between treatments was determined by one-way ANOVA taking $p<0.05$ as significant

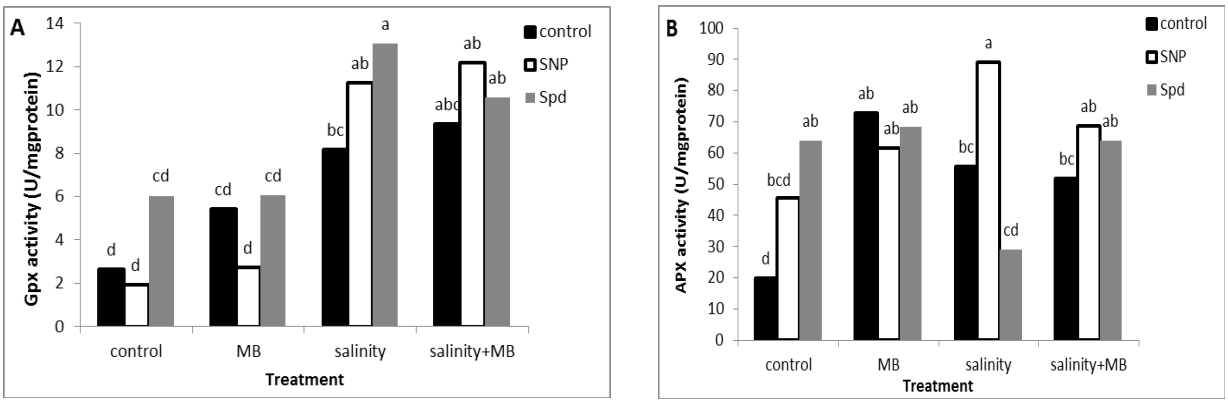

Figure 3. Effect of SNP, Spd and MB treatments on the activity of GPX (A) and APX (B) activity in chamomile plant leaves under control and salt stress condition. Data are means ± SE of three replicates. The significant of different between treatments was determined by one-way ANOVA taking $p<0.05$ as significant.

DISCUSSION

Salt stress disturbs intracellular ion homeostasis of plants, which leads to membrane dysfunction, attenuation of metabolic activity, and cause growth inhibition and ultimately leads to cell death (Sheokand *et al.,* 2010). A key factor limiting plant growth is excessive Na^+, a harmful mineral element not required by most plants. Our finding showed that salinity stress has the negative effects on shoot and root length and pre-treatment with SNP or Spd decreased NaCl damages, supporting that NO and Spd is actively involved in the regulation of plant growth. However, application of SNP and Spd with MB declined the improvement effect of SNP and Spd on plant growth under salinity stress. Previous studies have demonstrated that the exogenous NO and polyamines mitigated decrease in plant growth caused by salinity is through increasing antioxidant system, alleviating oxidative damage (Shi *et al.*, 2007; Zheng *et al.,* 2011) and stimulating vacuolar H^+-ATPase and H^+-PPase activities (Liu *et al*., 2006) etc. Salt stress induces lipid peroxidation by production of ROS (Shi *et al.*, 2007 and Zhang *et al.*, 2004), thus making the membranes leaky as evinced by increase electrolyte leakage. The membrane injury was time dependent and increased with duration of stress. In the present investigations, NaCl significantly increased the MDA content, while SNP and Spd alleviate the adverse effect of NaCl on MDA concentration in chamomile plants. A protective effect of NO on membrane injury has been reported under salt (Zhao *et al.*, 2004), drought (Nasibi and Kalantari, 2009) and heavy metal stress (Singh *et al.*, 2008). It has been reported that role of NO in suppression of lipid peroxidation probably is related to NO reaction with radicals of lipid alcoxyl ($LO^{\cdot}$) and lipidperoxyl ($LOO^{\cdot}$) that suppressed chain of peroxidation (Beligni and Lamattina, 1999), that compatibility with results of this experiment about reduction of MDA content by NO pretreatment. The role of polyamines in decline of MDA content also had been reported in some of plants under stress condition (Velikova *et al.*, 2000 and Tang and Newton, 2005). The defensive role of polyamines may be related to the nature of these compounds, which can act as anti- oxidant, snatcher of free radicals and membrane fixator (Velikova *et al.*, 2000). In this investigation, pretreatment of plants with combination of SNP with MB decrease the defensive effect of NO on lipid peroxidation, but in plant which were pretreated with (Spd+ MB),

MDA content did not change significantly in comparison with plants treated with Spd. Reduction of defensive effect of NO on lipid peroxidation by MB presumably concerned to the effect of MB on inhibition of signaling pathway of NO. Under normal conditions, the total amount of ROS formed in the plants is determined by the balance between the multiple ROS producing pathways and the ability of the enzymatic and non-enzymatic mechanism to deal with them. Under stress conditions, ROS formation is higher than ability of plants to remove it, and this could result in oxidative damages (Laspina *et al.*, 2005). In chamomileplants under salinity, APX and GPX activities were elevated over the controls,therefore, we can assume that the plant antioxidant machinery was effectively struggling against stressful condition. Relatively higher activities of ROS-scavenging enzymes have been reported in tolerant genotypes when compared to susceptible ones, suggesting that the antioxidant system plays an important role in plant tolerance against environmental stresses (Shi *et al.*, 2007). In addition, the results showed that under salt stress H_2O_2 content increased (Fig. 2). Increment of H_2O_2 content under salt stress was reported in response to salt stress in previous research (Uchida *et al.*, 2002). Pretreatment with SNP and Spd decreased the H_2O_2 content and alleviated the salinity stress. In this study, the activity of GPX enzyme was significantly increased by exogenous Spd treatment, while SNP pretreatment is caused significant increase the activity of APX activity. In chamomile plants that exposed salt stress, the combination of MB with SNP (MB + SNP) and Spd (MB + Spd) had no significant effect on GPX activity, but (MB + Spd) treatment caused significant increase APX activity compared to plants treated with Spd alone. A protective role of NO on H_2O_2 content has been reported under water stress (Zhao *et al.*, 2008), salt stress (Sheokand *et al.*, 2010) and heavy metal stress (Singh *et al.*, 2008). It was suggested when NO/O_2^- proportion 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 (Dellendonne *et al.*, 2001). Peroxynitrite has been shown to combine with H_2O_2 to produce nitrite ion and oxygen (Beligni and Lamattina, 2001). In many studies, it was found that the function of PA alleviation of oxidative stress was attributed to induction of various ROS-scavenging enzyme activities (Hsu and Kao 2007; Wang *et al.*, 2007; Tang *et al.*, 2005). Combination of MB with SNP (SNP + MB) and Spd (Spd + MB) had not significant effect on H_2O_2 content in chamomile plants under stress condition. The result of this study indicated that MB could partially and entirely abolish the protective effect of NO on some physiological parameters under stress condition. Methylene blue also could reduce the alleviation effect of Spd on some of parameters in chamomile plant under salt stress. Comparing the effects of NO and SNP in this study showed that Spd probably acts through NO pathway, but the use of PTIO(scavenger of NO) is better to prove.

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