The investigation of the alleviated effect of copper toxicity by exogenous nitric oxide in tomato plants

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ABSTRACT

As a bioactive signal, nitric oxide (NO) is involved in multiple plant physiological responses, especially under some abiotic stress. Here, we investigated the effects of exogenous nitric oxide on both the reactive oxygen species (ROS) scavenging metabolism and regulating functions of plasma membrane and tonoplast in tomato plants treated with 50μM $CuCl_2$. Copper stress induced significant accumulation of H_2O_2, led to serious lipid peroxidation, and finally markedly decreased shoot height and fresh weight of tomato plants. The application of 100μM sodium nitroprusside (SNP – NO donor) promoted some antioxidant enzymes, reduced accumulation of H_2O_2, and adjusted the activity of H^+-ATPase and H^+-PPase in plasma membrane or tonoplast, and significantly alleviated the growth inhibition induced by copper toxicity. On the other hand, the application of sodium ferrocyanide (an analog of SNP) and sodium nitrate or nitrite (the decomposition product of NO or its donor SNP) which did not release NO, did not show the effects of SNP. Furthermore, the effects of SNP were reverted by addition of hemoglobin (an NO scavenger). Therefore, these results indicated that exogenous NO could effectively assuage copper toxicity by physiological and biochemical response so as to maintain normal growth.

Keyword: nitric oxide (NO); copper toxicity; tomato; enzyme activity; plasma membrane; tonoplast

Copper is an indispensable element for growth and development of plants. Like most other micronutrients, copper is needed in small amounts (Tanyolac et al. 2007). Yet, as a kind of heavy metal, copper is toxic even at very low concentration. Recently, copper has become increasingly hazardous due to its involvement among pollutants of agricultural soils, in fungicides, pesticides, fertilizers, animal dung and so on (Kaplan 1999). In spite of its physiological importance, an increase in Cu content threatens plant health because excessive copper would cause destruction of the lipid and protein constituent of membrane (Maksymiec 1997), even if mildly excessive. The destruction of membrane was reported to result from the overproduction of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), hydroxyl radical (OH) and superoxide radicals (O_2^-) directly (Mazhoudi et al. 1997, Apel and Hirt 2004). To counteract the toxicity of copper, plants have developed various strategies by exudation of organic acid, activating antioxidant enzymes, retention of copper in roots and immobilization in the cell wall.

Nitric oxide (NO) is a small, highly diffusible gas and a ubiquitous bioactive molecule. Its chemical properties make NO a versatile signal molecule that functions through interaction with cellular targets via either redox or additive chemistry (Lamattina et al. 2003). The effects of NO, in some cases, are the results of its interaction with ROS. These interactions could be sometimes cytotoxic or cytoprotective depending on the concentration and situation (Wink and Mitchell 1998). It has been widely accepted that NO protects plant cell against oxidative stress by scavenging Fenton active Fe and regulating activities of antioxidant enzymes (Wink et al. 1995, Arasimowicz et al. 2007). However, a large amount of NO may combine with O_2^- to form peroxynitrite ($ONOO^-$), which has been reported to damage lipids, proteins and nucleic acids (Lipton et al. 1993, Yamasaki et al. 1999). Nevertheless, O_2^- and H_2O_2 are more toxic than $ONOO^-$.

Supported by the Young Scientist Encouraging Foundation of the Shandong province, Project No. BS2009HZ016.

NO has been reported to counteract the toxicity of ROS generated by excessive cadmium (Hsu and Kao 2004, Laspina et al. 2005), excessive copper (Yu et al. 2005, Tewari et al. 2008), salt stress (Zhao et al. 2004, Shi et al. 2007), and dramatically promote the germination of wheat seeds under copper stress (Hu et al. 2007). Particularly, alleviatory role of NO in copper toxicity is still scanty and needs more attention. Therefore, the objective of this experiment was to investigate physiological and biochemical mechanism of exogenous NO increasing tomato tolerance to copper toxicity.

MATERIAL AND METHODS

Plant materials and experimental design. Tomato seeds (*Lycopersicon esculentum* Mill, cv. Meigui) were sterilized with 55°C distilled water for 30 min, and germinated on moist filter paper in the dark at 28°C for 2–3 days. The germinated seeds were planted in vermiculite and grown in greenhouse for 8 days. Then the seedlings were cultivated with half-strength Hoagland's solution. After two weeks tomato seedlings were transplanted into perforated polystyrene plates, floating on an aerated Hoagland nutrient solution renewed every three days. Pre-cultivated for three weeks, uniform size seedlings were selected and transplanted into 5000 ml black plastic containers with five seedlings per container. The experimental design is given in Table 1. Sodium nitroprusside (SNP) was an NO donor, while sodium ferrocyanide was an analog of SNP. Sodium nitrate or nitrite was the decomposition product of NO or its donor SNP, which did not release NO and hemoglobin was an NO scavenger. The treatments were arranged in a randomized, complete block design with three replicates. Daily photoperiod of 12–14 h was given to plants and the air temperature was 16–25°C day and night. pH was adjusted to 5 ± 0.2 with HCl or KOH. Growth parameters and enzyme activity were measured after 8 days of copper treatment.

H_2O_2 and MDA content assay. H_2O_2 content was determined according to Patterson et al. (1984). The level of lipid peroxidation was measured in terms of malondialdehyde content by the thiobarbituric acid reaction method (Heath and Packer 1968).

Antioxidant enzyme extraction and assay. For extraction of antioxidative enzymes, fresh leaves and roots were homogenized with 50mM Na_2HPO_4-NaH_2PO_4 buffer (pH 7.8) containing 0.2mM EDTA and 2% insoluble polyvinylpyrrolidone in a chilled pestle and mortar. The homogenate was centrifuged at 12 000 × g for 20 min and the resulted supernatant was used for determination of enzyme activities. The whole extraction procedure was carried out at 4°C. All spectrophotometric analysis was conducted on a Shimadzu UV-2450 spectrophotometer. SOD activity was assayed according to Rao and Sresty (2000), POD activity according to Adams (1978), CAT activity according to Cakmak and Marschner (1992), APX activity according to Nakano and Asada (1981).

Isolation of plasma membrane and tonoplast vesicles. Plasma membrane and tonoplast vesicles fraction-enriched were isolated from tomato roots and leaves as described by Kasamo (1986) and Yu et al. (1997) with minor changes. Excised roots and leaves were homogenized (1/2, w/v) with a mortar and pestle in a cold grinding medium containing: 25mM HEPES-Tris (pH 7.2), 250mM mannitol, 5mM EDTA, 5mM EGTA, 1mM DTT and 1.5% (w/v) PVP. The whole isolation procedures were carried out at 4°C. The homogenate was filtered through four layers of cheesecloth and centrifuged at 560 × g for 12 min, then the supernatant was centrifuged at 10 000 × g for 15 min, and the supernatant was centrifuged at 60 000 × g for 30 min to yield a crude membrane fraction. The resulting pellet was resuspended with 1 ml in a gradient buffer containing: 20mM HEPES-Tris (pH 7.5), 5mM EDTA, 0.5mM EGTA. The supernatant was layered on top of a step gradient consisting of 1 ml of 45%, 33% and 15% (w/w) sucrose, respectively, and then centrifuged for 2 h at 70 000

Table 1. The experimental design

No.	Treatments
Control	no added SNP and $CuCl_2$
Cu	50μM $CuCl_2$
Cu + SNP	50μM $CuCl_2$ + 100μM SNP
Cu + N	50μM $CuCl_2$ + 100μM sodium nitrite + 100μM sodium nitrate
Cu + F	50μM $CuCl_2$ + 100μM sodium ferrocyanide
Cu + SNP + Hb	50μM $CuCl_2$ + 100μM SNP + 0.1% bovine hemoglobin

× g. The tonoplast-enriched fraction was collected at the 15/33% sucrose interface and the plasma membrane-enriched fraction was collected at the 33/45% sucrose interface. Each fraction was centrifuged for 1 h at 100 000 × g. The resulting pellet was resuspended in a medium containing: 20mM HEPES-Tris (pH 7.5), 3mM $MgCl_2$, 0.5mM EGTA, 300mM sucrose, quickly frozen in liquid N_2 and stored at –70°C until used for the enzyme assays.

Measurement of H^+-ATPase in PMs. ATP hydrolysis assays were performed as described by Briskin et al. (1987). 0.5 ml the reaction medium containing: 36mM Tris-Mes (pH 6.5), 30mM ATP-Na_2, 3mM $MgSO_4$, 1mM NaN_3, 50mM KNO_3, 1mM Na_2MoO_4, 0.02% (v/v) Triton X-100, in the presence or absence of 2.5mM Na_3VO_4. The reaction was started by adding 50 µl PM vesicles. After 30 min incubation at 37°C, the reaction was quenched by the addition of 55% (w/v) TCA. The H^+-ATPase activity was determined by measuring the release of Pi (Ohinishi et al. 1975).

Measurement of H^+-ATPase and H^+-PPase activities in tonoplast. H^+-ATPase and H^+-PPase activities were calculated as the rate of ATP hydrolysis or PPi, respectively, by measuring the amount of released Pi according to Lin and Morales (1977). Activities of tonoplast H^+-ATPase were measured in a 0.5 ml reaction medium containing: 36mM HEPES-Tris (pH 7.5), 30mM ATP-Na_2, 3mM $MgSO_4$, 0.5mM NaN_3, 50mM KCl, 0.1mM Na_2MoO_4, 0.1mM Na_3VO_4, in the presence or absence of 50mM KNO_3. Activities of tonoplast H^+-PPase were measured in a 0.5 ml reaction medium containing: 60mM Tris- HEPES (pH 8.0), 3mM $MgSO_4$, 5mM EDTA, 1mM $(NH_4)_6Mo_7O_{24}$, 0.5mM NaN_3, 0.1mM Na_3VO_4, 30mM Na-pyrophosphate, in the presence or absence of 100mM KCl. The reaction was started by adding 50 µl tonoplast vesicles. After 30 min incubation at 37°C, the reaction was quenched and the color developed as in the PM ATPase assay.

Copper content assay. Leaf and root samples were oven dried at 70°C, until they reached a constant weight. The dried plant material was ashed at 480°C in a muffle furnace, twice digested in 10 ml of 3M HCl at 90°C, and analyzed by AA370MC flame atomic absorption spectrophotometry.

Data statistic. All data presented were means ± one standard deviation (S.D.) of three replicates. Statistical analyses were performed by analysis of variance (ANOVA) using the SAS software. Differences between treatments were separated by the least significant difference (LSD) test at a 0.05 probability level.

RESULTS

Hydrogen peroxide content and lipid peroxidation. Compared to control, Cu treatment significantly increased H_2O_2 content in tomato roots and leaves. The application of exogenous NO dramatically decreased accumulation of H_2O_2 under copper stress, which was blocked by addition of hemoglobin. Addition of sodium ferrocyanide and sodium nitrate or nitrite had no significant effect on H_2O_2 content under copper stress (Figure 1A).

When plants were subjected to environmental stress, oxidative damage resulted in membrane lipid peroxidation, which could be estimated by MDA content. Similar to H_2O_2 change, Cu treatment significantly increased MDA content in tomato

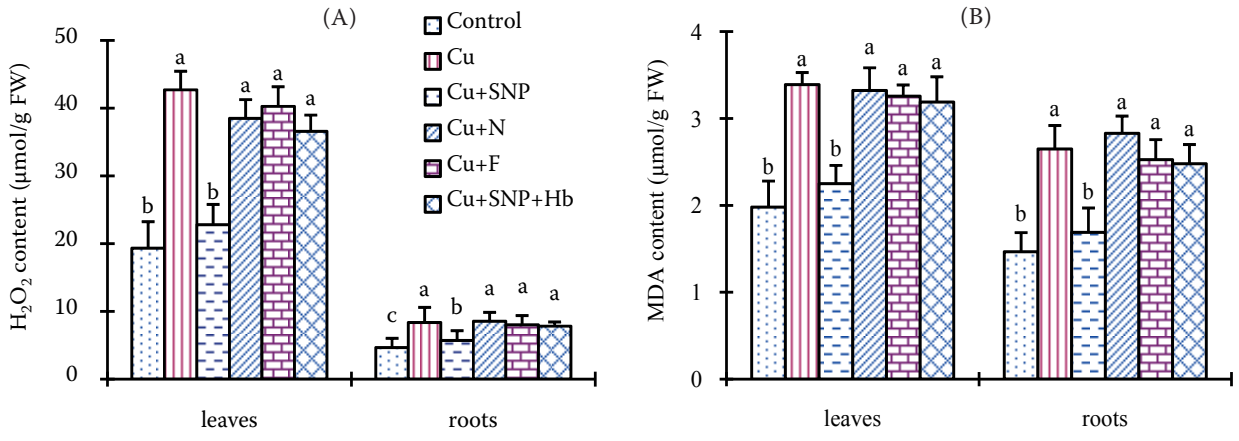

Figure 1. Effects of exogenous NO supply on H_2O_2 content (A), MDA content (B) in leaves and roots of tomato plants

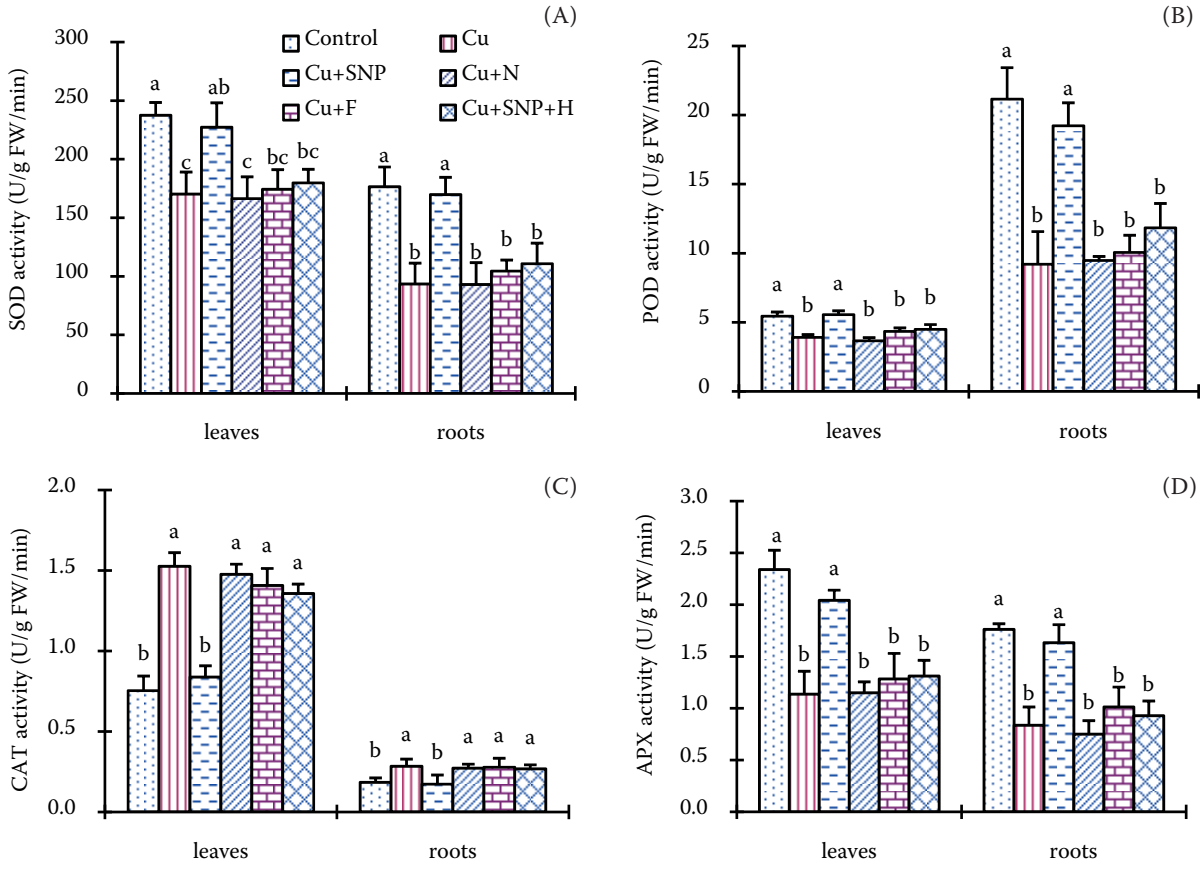

Figure 2. Effects of exogenous NO on activities of SOD(A), POD(B), CAT(C), APX(D) in leaves and roots of tomato

leaves and roots, while exogenous NO significantly reduced MDA content. And the alleviated effect was also removed by addition of hemoglobin. Application of sodium ferrocyanide and sodium nitrate or nitrite did not markedly affect MDA content (Figure 1B).

Antioxidant enzymes. It can be seen that in the leaves and roots of tomato plant, SOD activity was significantly decreased by Cu stress, and in roots the inhibition was significantly alleviated by exogenous NO supply, the addition of hemoglobin removed the alleviated effect, and application of sodium ferrocyanide and sodium nitrate or nitrite did not change the SOD activity in copper-treated plants. In tomato leaves, exogenous NO supply elevated SOD activity, yet, it did not reach to statistically significant differences (Figure 2 A).

Cu treatment significantly inhibited the activity POD and APX in both leaves and roots of tomato, and exogenous NO supply greatly decreased the inhibition, while the addition of hemoglobin removed the effect of exogenous NO, and addition of sodium ferrocyanide and sodium nitrate or nitrite did not obviously change the tendency (Figures 2 B and D).

Contrary to the change of POD and APX activity, CAT activity in both leaves and roots was markedly enhanced on the 8th day of treatment with copper stress. Exogenous NO significantly decreased CAT activity under copper stress. Moreover, application of hemoglobin with SNP, sodium ferrocyanide and sodium nitrate or nitrite did not significantly influence CAT activity (Figure 2 C).

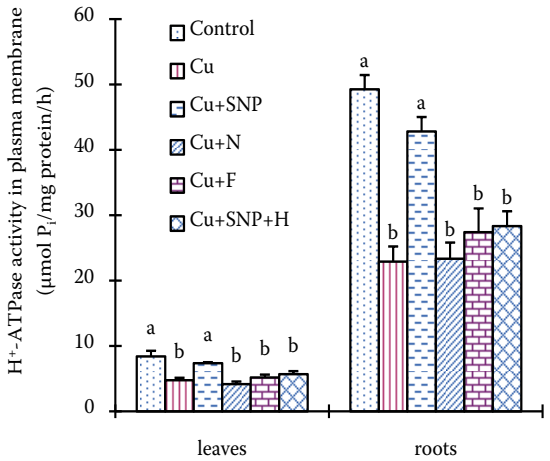

Figure 3. Effects of exogenous NO supply on activity of H^+-ATPase in plasma membrane of tomato leaves and roots

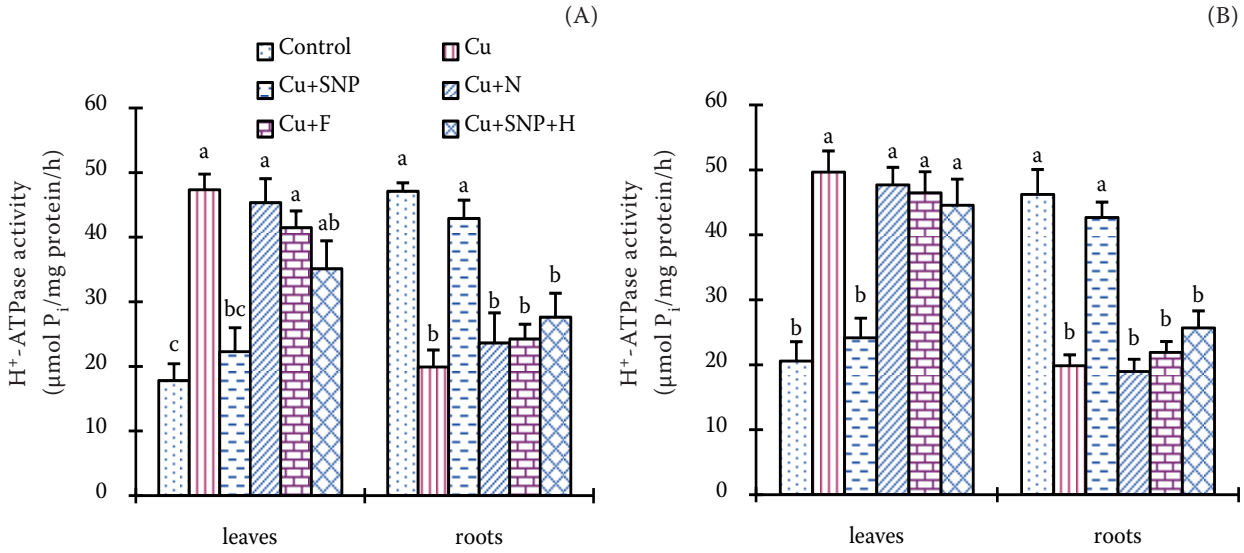

Figure 4. Effects of exogenous NO supply on the activity of H^+-ATPase (A), H^+-PPase (B) in tomato tonoplast

H^+-ATPase activity in plasma membrane. As shown in Figure 3, copper stress markedly inhibited H^+-ATPase activity both in leaves and roots, and exogenous NO supply dramatically decreased the inhibition. Addition of sodium ferrocyanide, sodium nitrate or nitrite and SNP with hemoglobin had no significant effect on H^+-ATPase activity in PMs.

H^+-ATPase and H^+-PPase activity in tonoplast. H^+-ATPase and H^+-PPase activity in both tomato leaves and roots tonoplast was also measured. In leaves and roots, their activities showed a different tendency. In leaves, copper stress markedly promoted tonoplast H^+-ATPase and H^+-PPase activities, while exogenous NO supply remarkably reduced the activities. The activity did not show statistically significant differences among Cu, Cu + F, Cu + N, and Cu + S + H treatments. However, in roots, the variable trend of enzyme activity was even contrary to the leaves.

Copper content. As shown in Figure 5, Cu content in both leaves and roots was significantly increased by Cu stress compared to control, while exogenous NO supply could not markedly change Cu content. This observation suggested that exogenous NO supply did not influence Cu uptake and transport. Cu content in roots varied more extensively than in leaves, which indicated that excessive copper inclines to accumulate in roots.

Plant growth. As shown in Figure 6, shoot height and fresh weight of both shoots and roots of tomato plants were significantly decreased by Cu stress compared to control, and the inhibition was significantly alleviated by exogenous NO supply. The alleviating effect could be blocked by hemoglobin. Moreover, Cu + F and Cu + N had no obvious effect on alleviation of copper toxicity.

DISCUSSION

It has been reported that Cu stress could cause an increased production of H_2O_2 (Weckx and Clijsters 1996) and induce membrane lipid peroxidation (Jouili and Ferjani 2003). These authors revealed that excessive Cu greatly induced a serious membrane damage in the plant. This experiment showed that oxidative stress of tomato induced by excessive Cu was effectively alleviated by application of SNP, while the application of sodium ferrocyanide, sodium nitrate or nitrite and SNP with hemoglobin did not mitigate the oxidative

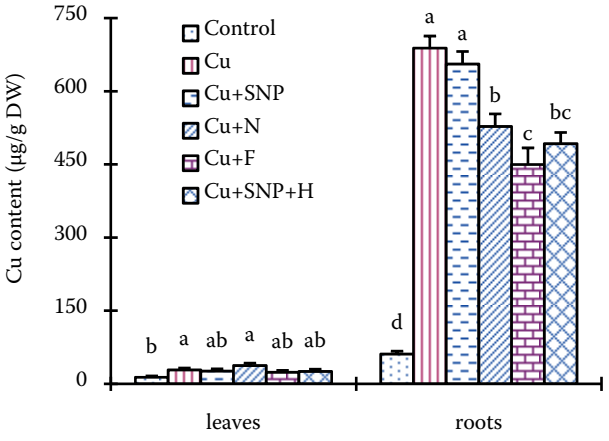

Figure 5. Effects of exogenous NO supply on Cu content in leaves and roots of tomato

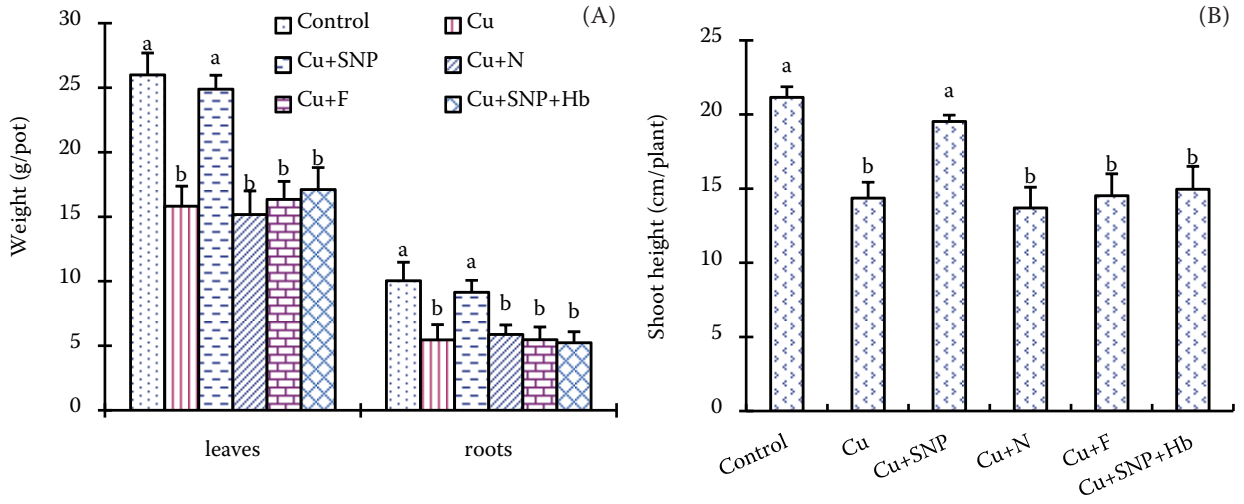

Figure 6. Effects of exogenous NO on shoot and root fresh weight (A) and shoot height (B) of tomato

damage. Therefore, by comparing the results, we could deduce that SNP alleviating copper toxicity was ascribed to NO release. In animals, NO has been reported to block the oxidative stress associated with NO, including modulating antioxidant enzyme activity to exert protective functions (Bogdan 2001). Also, Tewari et al. (2008) reported NO could significantly attenuate Cu-induced oxidative damage as indicated by a down regulation in lipid peroxidation and H_2O_2 concentration in adventitious roots of mountain ginseng.

As key elements in the defense mechanisms, varying reactions of antioxidative enzymes have been observed under Cu stress conditions in many plants. For example, it was reported that ROS-scavenging enzyme activity increased induced by excessive Cu in maize (Tanyolac et al. 2007), barley plant (Demirevska-Kepova et al. 2004), tomato (Martins and Mourato 2006), bean shoots (Weckx and Clijsters 1996) and duckweed (Teisseire and Guy 2000), but decreased in rice leaves (Chen and Kao 1999), or were unaffected as in tomato roots and stems (Mazhoudi et al. 1997). In the present experiment, activities of SOD, POD and APX in tomato roots and leaves were significantly inhibited by Cu stress, while CAT activity was increased. This result implies that CAT induction might play an important role in tomato tolerance to Cu excess. Some of the observed results were consistent with the reports of Martins and Mourato (2006), and some were not consistent with them. The difference indicated that the influence of Cu stress on the antioxidant enzymes were very complex and related to the plant treatment time, plant tissues, plant species and genotypes.

In many studies, it was reported that the function of NO alleviation of oxidative damage was ascribed to induction of various ROS-scavenging enzyme activities (Hsu and Kao 2004, Hu et al. 2007). In the present work, application of SNP dramatically decreased the SOD activity inhibited by the Cu stress; it suggests that application of NO could promote the conversion from O_2^- into H_2O_2 and O_2, which is an important step in protecting the cell. If the generated H_2O_2 could not be scavenged efficiently, it could interact with O_2^- to form highly reactive hydroxyl radicals (OH) that were thought to be primarily responsible for oxygen toxicity in the cell (Shi et al. 2007). Therefore, the efficient scavenging of H_2O_2 was very important for normal metabolism of plant. Exogenous NO greatly induced the H_2O_2-scavenging enzymes POD and APX under Cu stress. Similar results were observed in the experiment of NO increasing the rice leaves tolerance to Cu excess (Yu et al. 2005) and in the experiment of NO stimulating wheat seed germination under Cu stress (Hu et al. 2007).

Plasma membrane H^+-ATPase is a P-type proton pump in plant. The transmembrane electrochemical gradient generated by the enzyme is the primary force for ions' cross-membrane transports (Michelet and Boutry 1995). In this study, Cu stress significantly inhibited PM H^+-ATPase in tomato plant. This might be attributed to oxidative stress induced by excessive Cu, because the optical activity of PM H^+-ATPase needed a specific lipid environment, while the lipid content and composition of plasma membrane also depended on the generation of free radicals (Gardner 1991, Rodriguez et al. 1999). Such as hydroperoxides of

polyunsaturated fatty acid, the process has undergone a variety of reactions including membrane properties, ultimately resulting in the dysfunction of the lipid bilayer and membrane deterioration (Gardner 1991). Ouariti et al. (1997) reported that significant decreases in the content of lipid classes and changes of fatty acid composition were recorded in Cu- and Cd-stressed tomato plants in comparison with controls. Application of NO significantly stimulated PM H^+-ATPase in tomato plant, which might be ascribed to optical lipid environment and be one mechanisms of NO increasing tomato tolerance to copper stress.

Under heavy metal and salt stress, transport of Zn^{2+}, Cd^{2+} and Na^+ from the cytoplasm into the vacuole via the tonoplast is a possible survival strategy of plants (Leach et al. 1990, Vazquez et al. 1994). V-H^+-ATPase and V-H^+-PPase could establish an electrochemical H^+-gradient across the tonoplast and maintain the concentration gradient. In the present study, H^+-ATPase and H^+-PPase activities of tonoplast in tomato leaves were increased, which implies that V-H^+-ATPase and V-H^+-PPase activities in tomato leaves induction might play an important role in tomato leaves tolerance to copper stress. V-H^+-ATPase and V-H^+-PPase activities were greatly inhibited under copper stress in roots, but were significantly elevated by the application of NO. The results indicated that less severe tonoplast damage occurred in NO-treated roots of tomato by excessive copper, and this might be also ascribed to the alleviation of ROS under Cu stress by NO-enhanced activities of antioxidant enzymes. It was well known that the degree of lipid peroxidation was closely related to the accumulation of ROS, and the lipid process was one important factor exerting an effect on ATPase under changing environmental conditions (Veselov et al. 2002). As a result of lipid peroxidation induced by Cu excess, ATPase and PPase proteins were disturbed. Therefore, exogenous NO decreasing lipid peroxidation should be considered as a cause maintaining higher ATPase activity.

The results of this study showed that exogenous NO was able to improve shoot height and fresh weight of tomato plants subjected to Cu stress. The lower level of H_2O_2 and MDA of Cu + SNP treatment showed that NO displayed antioxidative activity. By determining the activities of SOD, POD, APX and CAT, we learned that NO exerted its protective effect through the activation of some antioxidative enzymes, not by preventing Cu uptake and transport. Therefore, lower lipid peroxidation could maintain better functioning of the plasma membrane and tonoplast, elevate the activities of PM H^+-ATPase, V-H^+-ATPase, V-H^+-PPase, and mitigate copper toxicity in tomato.

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Received on May 17, 2009

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