ORIGINAL ARTICLE

Exogenous nitric oxide mediates alleviation of mercury toxicity by promoting auxin transport in roots or preventing oxidative stress in leaves of rice seedlings

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Abstract Nitric oxide (NO), a multifunctional gaseous molecule, mediates a variety of responses to biotic and abiotic stresses. The effects of exogenous NO on rice (*Oryza sativa* cv. 'Zhonghua 11') growth under mercuric chloride ($HgCl_2$) stress were investigated. The results showed that 60 μM Hg significantly inhibited the root elongation of rice plantlets after seed germination. While 100 μM or 200 μM sodium nitroprusside (SNP, a donor of NO) could increase the root length by attenuating the effects of 2,3,5-triiodobenzoic acid (TIBA) and Hg, which indicated the role of NO in auxin transport-promoting in roots. On the other hand, SNP decreased the absorption and transportation of Hg in roots and shoots of rice seedlings at five-leaf stage. Moreover, the levels of superoxide radical ($O_2 \cdot^-$) and hydrogen peroxide (H_2O_2) in leaves were also decreased significantly. However, the activities of antioxidant enzymes were not enhanced by SNP. Moreover, NO promoted the growth of rice plantlets under Hg stress even when superoxide dismutase (SOD, EC 1.15.1.1) or catalase (CAT, 1.11.1.6) activity was inhibited by diethyldithiocarbamate (DDC, an inhibitor of SOD) or 3-amino-1,2,4-triazole (AT, an inhibitor of catalase), respectively. These results confirmed that NO could act as the direct quencher of $O_2 \cdot^-$ and then prevent the oxidative damage caused by Hg ion in leaves.

Keywords Nitric oxide (NO) · Mercury stress · Oxidative stress · Auxin transport · $O_2 \cdot^-$ quencher · *Oryza sativa*

Abbreviations

Hg	Mercury
NO	Nitric oxide
SNP	Sodium nitroprusside
ROS	Reactive oxygen species
$O_2 \cdot^-$	Superoxide radical
H_2O_2	Hydrogen peroxide
SOD	Superoxide dismutase
CAT	Catalase
DDC	Diethyldithiocarbamate
AT	3-amino-1,2,4-triazole
TIBA	2,3,5-triiodobenzoic acid

Introduction

Mercury (Hg) is a high-risk global pollutant to public health because of the potent neurotoxicity (Chen and Yang 2012; Sunderland and Selin 2013). Inputs of Hg by anthropogenic activities and Hg by-product emission into the environment have reached as much as 3×10^6 kg per year (Kim and Jung 2012). Hg is easily taken up by plants due to its transitional properties, induces the generation of reactive oxygen species (ROS) and then causes oxidative damage (Cho and Park 2000; Cargnelutti et al. 2006; Shiyab et al. 2009; Gao et al. 2010; Sahu and Sahoo 2012). Hg could also inhibit the root growth and development, and

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then affect the water metabolism and mineral nutrition (Chen et al. 2012). But the mechanism and molecular process still remain elusive.

Nitric oxide (NO), a bioactive gaseous molecule, has been proven to play a prominent multifunctional signaling role in mediating a variety of physiological processes and responses to biotic and abiotic stresses (Besson-Bard et al. 2008; Fernández-Marcos et al. 2012; Abat and Deswal 2013). Just as was discovered more recently, NO appears to be involved in the regulation of heavy metal-induced oxidative stress and plant tolerance to heavy metals (Singh et al. 2009; Xiong et al. 2009a, b; Saxena and Shekhawat 2013). For example, NO could depress the generation of hydrogen peroxide (H_2O_2) and alleviate Al toxicity by enhancing antioxidative capability (Wang and Yang 2005) or by regulating hormonal equilibrium (He et al. 2012). Zhang et al. (2008) reported that generation of endogenous NO was positively associated with the proline level in Cu-treated algae. Furthermore, NO could alleviate phytotoxicity by directly regulating accumulation and translocation of heavy metals in plants. Application of sodium nitroprusside (SNP, a donor of NO) was able to reduce root-to-shoot translocation of Ni in *Brassica napus* (Kazemi et al. 2010). The detoxification by NO also might be related to the modulation of cell wall components, pectin and hemicelluloses (Xiong et al. 2009a; Zhang et al. 2012). However, little information is available regarding the role of NO in regulating the Hg-induced stress in rice. In the present study, we prove that NO is able to alleviate the Hg-induced inhibition of root growth or oxidative stress in rice seedlings.

Materials and methods

Plant materials, growth conditions and treatments

Seeds of rice (*Oryza sativa* cv. 'Zhonghua 11') were surface-sterilized with 20 % (v/v) sodium hypochlorite (NaClO) for 20 min, rinsed thoroughly with distilled water and soaked at 37 °C in dark for 2 days. Then each of the ten germinated seeds were transferred onto sterilized solid medium (1 % agar) supplemented with different chemical reagents as follows: CK (SNP 0 μM, $HgCl_2$ 0 μM), SNP 100 μM, SNP 200 μM, $HgCl_2$ 60 μM, $HgCl_2$ 60 μM + SNP 100 μM, $HgCl_2$ 60 μM + SNP 200 μM, $HgCl_2$ 60 μM + KNO_2 200 μM, $HgCl_2$ 60 μM + $K_4Fe(CN)_6$ 200 μM, $HgCl_2$ 60 μM + TIBA (2,3,5-triiodobenzoic acid, the polar transport inhibitor of IAA) 1 μM, or $HgCl_2$ 60 μM + TIBA 1 μM + SNP 200 μM, in transparent glass bottle and cultured vertically in a sterile environment. The photoperiod of the growth chamber was 13-h light (28 °C)/11-h dark (22 °C) with 80 % RH. After 7 days of treatments, the phenotype was observed and the length of shoot and root were measured.

For further observations and physiological experiments, other rice seedlings were cultured with the Yoshida's culture solution without any treatment until five-leaf stage (Yoshida et al. 1976). The culture solution was changed every 5 days. Then uniform seedlings were transferred to culture solutions containing SNP 100 μM, SNP 200 μM, $HgCl_2$100 μM, $HgCl_2$ 100 μM + SNP 100 μM, or $HgCl_2$100 μM + SNP 200 μM, and the treatment without any chemical reagent was set as control. After 3 days treatments, growth of seedlings was analyzed and chemical experiments as follows were performed.

Moreover, antioxidant enzyme inhibitors were applied for clarifying the mechanism of NO on antioxidant system. The emerge-germinating seeds were also treated as follows: $HgCl_2$ 60 μM + DDC (diethyldithiocarbamate, an inhibitor of Cu/Zn-SOD) 3 mM, $HgCl_2$ 60 μM + DDC 3 mM + SNP 200 μM, $HgCl_2$ 60 μM + AT (3-amino-1,2,4-triazole, an inhibitor of catalase) 2 μM and $HgCl_2$ 60 μM + AT 2 μM + SNP 200 μM.

Determination of Hg concentration

Roots of seedlings at five-leaf stage treated with Hg or Hg + SNP were immersed first in 20 mM Na_2-EDTA for 30 min and rinsed three times with deionized water. Then the roots and shoots of different treatments were collected respectively, dried at 105 °C for 20 min and then at 70 °C until a constant weight. Each plant material of 200 mg Dw was digested thoroughly with a mixture of HNO_3/HF (6/1, v/v) using multiwave digestion oven. The solution was filtered and diluted to the suitable concentration. Measurement of Hg concentration was carried out by atomic absorption spectrophotometer (AA-7000, Shimadzu, Japan) with hydride generator (HVG-1).

Detection of superoxide radical ($O_2\cdot^-$) and hydrogen peroxide (H_2O_2)

$O_2\cdot^-$ production rate was estimated by the method of detecting the nitrite formation from hydroxylamine in the presence of superoxide radical (Mishra and Singhal 1991). H_2O_2 was measured according to the previously described method (Jana 1981). Segment of leaves (0.2 g) was ground into powder in liquid N_2 and homogenized in 3 ml of pre-cooled 50 mM phosphate buffer (pH 6.5) with ice bath. The homogenate was centrifuged at 6000×*g* for 25 min. And then 1 ml of 0.1 % titanium sulphate in 20 % (v/v) H_2SO_4 was added to the above suspension. After that the mixture was centrifuged at 6000×*g* for 15 min. Then absorbance of the supernatant was recorded at 410 nm. H_2O_2 level was calculated using the extinction coefficient

0.28 μM^{-1} cm^{-1} and was expressed as nmol g^{-1} initial fresh weight.

Assays of antioxidant enzymes

Enzymes were extracted according to the reported method (Guo et al. 2007). 0.5 g of leaf tissue was homogenized in 5 ml ice-cold extraction buffer containing 50 mM potassium phosphate (pH 7.8), 0.2 mM EDTA and 2 % polyvinyl pyrrolidone (PVP-40, w/v). The homogenate was centrifuged at 10,000×*g* for 20 min at 4 °C. Then the supernatant was used as crude extract for further antioxidant enzyme assays.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed by measuring its inhibition of photochemical reduction of nitroblue tetrazolium chloride (NBT) described by Beauchamp and Fridovich (1971). The reaction mixture contained 50 mM potassium phosphate (pH 7.8), 0.1 mM EDTA, 67 μM NBT, 13 mM L-methionine, and 1.3 μM riboflavin and suitable aliquot of enzyme extract. Reactions were carried out at 30 °C under light intensity of about 200 μmol m^{-2} s^{-1} for 10 min. The absorbance of mixture was measured at 560 nm and one unit of SOD was defined as the amount of enzyme required to inhibit 50 % initial reduction of NBT under light.

The peroxidase (POD, EC 1.11.1.7) activity was measured following the method reported previously (Rao et al. 1997) with some modifications. 3 ml of the reaction mixture contained 100 mM potassium phosphate buffer (pH 6.0), 1.68 μl guaiacol, 1.14 μl H_2O_2 and 30 μl enzyme extract. The increase of absorbance at 470 nm for 2 min was recorded continuously to reflect the effect of POD. And the final POD activity was calculated using the extinction coefficient of 26.6 mM^{-1} cm^{-1} of tetraguaiacol formation.

The activity of catalase (CAT, EC 1.11.1.6) was determined in terms of the procedure described by Aebi (1983) with slight modifications. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.2), 50 μl enzyme extract and 10 mM H_2O_2. The decrease in absorbance at 240 nm was recorded due to the consumption of H_2O_2. In addition, the extinction coefficient for H_2O_2 is 39.4 mM^{-1} cm^{-1}.

The enzyme ascorbate peroxidase (APX, EC 1.11.1.11) was assayed according to the method as described by Nakano and Asada (1981). The decrease of absorbance at 290 nm was recorded and the extinction coefficient of 2.8 mM^{-1} cm^{-1} was used for ascorbate oxidation.

Statistical analysis

All experiments were performed in triplicate. Data points were presented as the mean ± standard deviation (SD). Significant differences between different treatments were analyzed by one-way analysis of variance (LSD test). SPSS 12.0 software (SPSS, Chicago, IL, USA) was used in all analyses.

Results

Exogenous NO alleviated Hg-induced inhibition on root length of rice plantlets after seeds germination

For primary test, the sterilized emerge-germinating seeds were treated with different reagents (Fig. 1) and $HgCl_2$ concentration of 60 μM was selected for this phase according to the pre-experiments. 7 days later, rice seedlings without any chemical stress grew well, and the average root (AR) length reached 6.23 cm. While a sharp reduction in root elongation was observed as Hg exposure, and the AR length was only 0.73 cm. SNP, either 100 μM or 200 μM, increased AR length significantly in comparison with the Hg treatment alone, and thereby effectively alleviated the inhibition caused by Hg stress.

In water solution, SNP decomposes spontaneously into NO, nitrite ion (NO_2^-) and ferrocyanide ion [$Fe(CN)_6^{4-}$]. As shown in Fig. 1, the results proved that the NO was primarily responsible for the SNP-induced beneficial effect, rather than the other compositions, KNO_2 and $K_4Fe(CN)_6$. On the other hand, results of treatments performed before with different concentrations confirmed that the effects of NO were dose dependent. Under the conditions without heavy metals stress, 1 μM and 10 μM SNP had slightly positive effects on the growth performance of rice plantlets. Then negative effects exerted with the increasing SNP concentrations (Xiong et al. 2009a). Nevertheless, 100 μM SNP and 200 μM SNP had better response on Cd detoxification (Xiong et al. 2009a, b). In accord with these results, 100 μM or 200 μM SNP alone inhibited root extension in part, but did recover the growth effectively for rice plantlets under Hg stress.

In order to elucidate the correlation of NO and auxin, TIBA, the inhibitor of auxin polar transport, was applied. And the research showed TIBA inhibited the lateral root formation, while SNP could obviously promote the lateral root formation and elongation, thus limiting the inhibitions caused by Hg and TIBA (Fig. 1).

Exogenous NO promoted the growth of rice seedlings at five-leaf stage under Hg stress

For further observation and physiological detections, the rice seedlings were cultured hydroponically until five-leaf

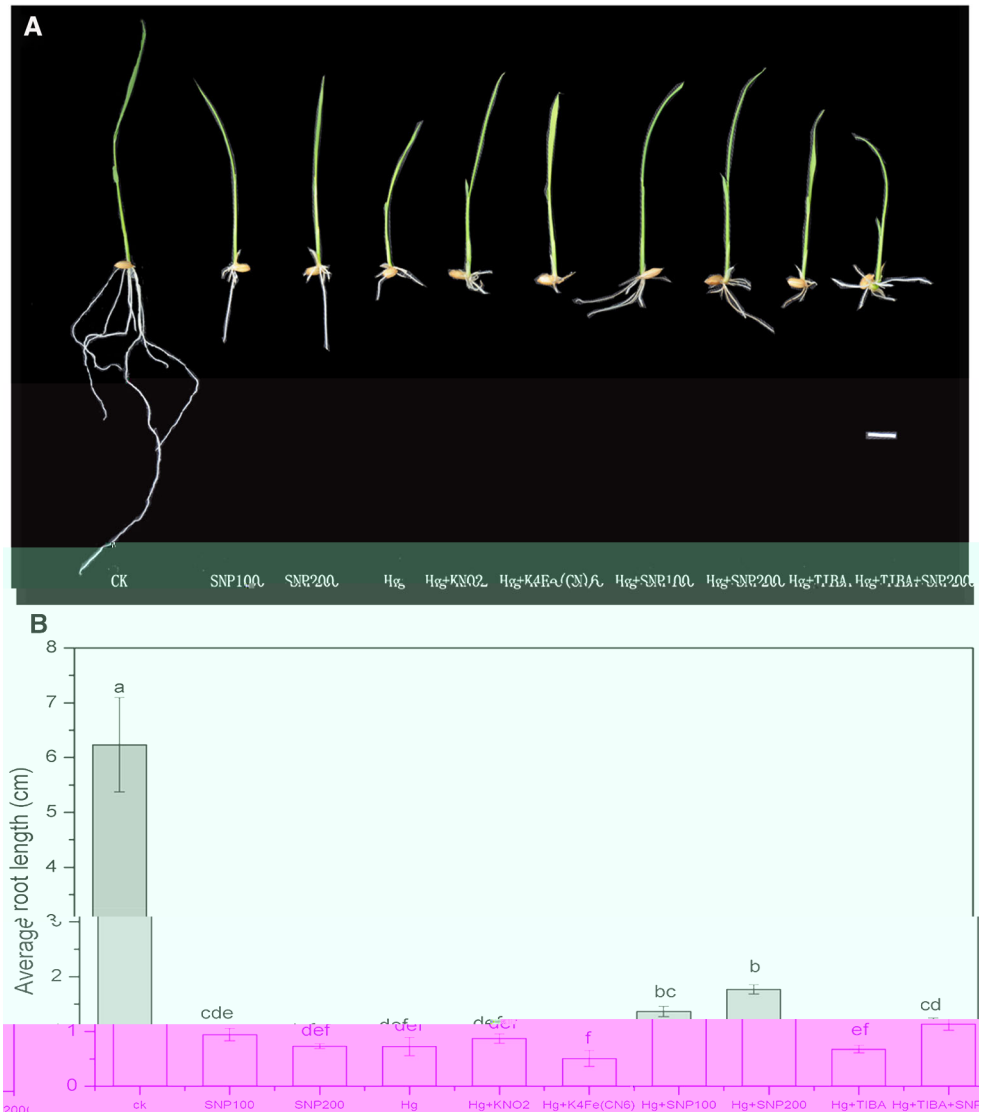

Fig. 1 Effects of SNP and its hydrolyzates on roots length of rice plantlets after germination under 60 μM $HgCl_2$ stress for 7 days by agar culturing in sterilized bottles. **a** Phenotypes of rice plantlets under different treatments: CK (SNP 0 μM, $HgCl_2$ 0 μM), SNP 100 μM, SNP 200 μM, $HgCl_2$ 60 μM, $HgCl_2$ 60 μM + SNP 100 μM, $HgCl_2$ 60 μM + SNP 200 μM, $HgCl_2$ 60 μM + KNO_2 200 μM, $HgCl_2$ 60 μM + $K_4Fe(CN)_6$ 200 μM, $HgCl_2$ 60 μM + TIBA 1 μM, or $HgCl_2$ 60 μM + TIBA 1 μM + SNP 200 μM; **b** Average root length of rice plantlets under different treatments. The *bar* presents 1 cm. Data are presented as the mean ± SD ($n = 10$). Values with different lowercase mean the significant difference at $P < 0.05$ level

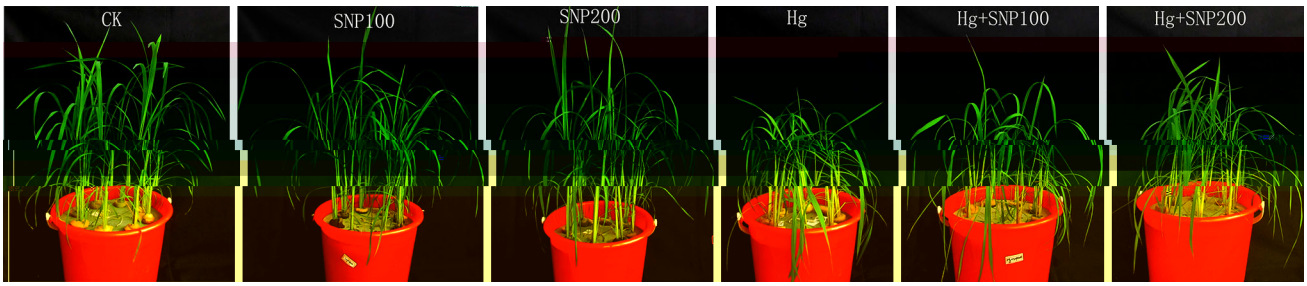

Fig. 2 Effects of SNP on growth of rice seedlings at five-leaf stage cultured hydroponically with or without 100 μM $HgCl_2$ treatments for 3 days. CK, SNP 0 μM, $HgCl_2$ 0 μM; SNP100, SNP 100 μM; SNP 200, SNP 200 μM; Hg, $HgCl_2$ 100 μM; Hg + SNP100, $HgCl_2$ 100 μM + SNP 100 μM; Hg + SNP200, $HgCl_2$ 100 μM + SNP 200 μM. The inner diameter of pail is 27 cm

stage and then they were treated with different reagents and higher concentration of $HgCl_2$. The seedlings became thoroughly chlorosis and necrosis after being exposed to 100 μM Hg stresses for 3 days (Fig. 2). But the symptoms of damage caused by Hg were notably relieved when SNP of 100 μM or 200 μM were added, indicating that the NO could mediate the alleviation of Hg toxicity and help the plants withstood the stress better.

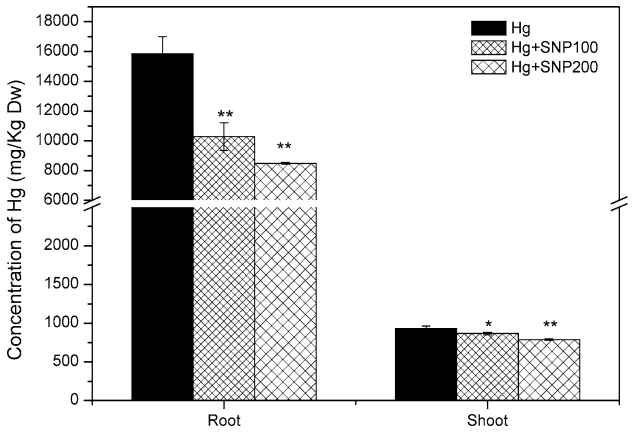

Fig. 3 Effects of NO (SNP 100 μM or 200 μM) on concentrations of Hg in rice seedlings at five-leaf stage cultured hydroponically with 100 μM $HgCl_2$ treatment for 3 days. Data are presented as the mean ± SD. The significant level of the difference between Hg and Hg + SNP treatment is indicated by $*P < 0.05$ or $**P < 0.01$

Exogenous NO regulated the uptake of Hg

The concentrations of Hg in different parts of plants under Hg or Hg + SNP treatments were analyzed (Fig. 3). Hg accumulated mainly in the roots of rice. However, the concentrations of Hg in roots of seedlings treated with Hg + SNP100 or Hg + SNP200 were significantly lower than that of Hg treatment alone, decreased by 35.1 and 46.4 %, respectively. So the absorption of Hg might be decreased by exogenous NO. And the transportation of Hg to shoots was also significantly inhibited by NO.

Exogenous NO decreased the levels of reactive oxygen species caused by Hg stress

Reactive oxygen species (ROS), including $O_2\cdot^-$, H_2O_2 and hydroxyl radical (·OH), have strong oxidative ability and often result in lipid peroxidation of biomembranes. SNP treatment alone did not induce excessive $O_2\cdot^-$ production in leaves of rice seedlings. On the contrary, exposure to Hg ion triggered a sharp increase of $O_2\cdot^-$ production rate. However, the supplements of SNP to solutions containing Hg reversibly lessened the $O_2\cdot^-$ levels.

H_2O_2 plays a dual role in plants: not only acts as a damaging agent, but also functions as a cellular messenger. Application of exogenous NO alone could promote the growth of rice seedlings obviously and the H_2O_2 in leaves of these plants kept at a low level. But the generation of H_2O_2 immediately reached a peak when $HgCl_2$ was exposed to the solution, and then recovered to the control level with the SNP supplements (Fig. 4).

Effects of NO on antioxidant enzyme activities

Under normal conditions, the antioxidant enzyme activities in leaves of rice seedlings were all at low levels. When subjected to Hg stress, the SOD, POD, CAT and APX were all significantly activated and their activities reached the peak levels (Fig. 5), but inhibited by addition of SNP. Under conditions of control, 100 μM SNP or 200 μM SNP treatments, the activities of SOD in leaves of plants were 13.37, 27.28 or 39.80 U mg^{-1} protein, respectively. However, it increased dramatically to 69.76 U mg^{-1} protein under Hg stress. SNP did not upregulate the SOD activity adequately and the SOD activity recovered to 29.04 or 39.84 U mg^{-1} protein with Hg + SNP100 or Hg + SNP200 treatment. The same tendency also occurred in other antioxidant enzymes detected in this study. All these factors indicated that 100 μM $HgCl_2$ could cause severe oxidative damage to rice seedlings and exogenous NO might act as a ROS scavenger as reported earlier

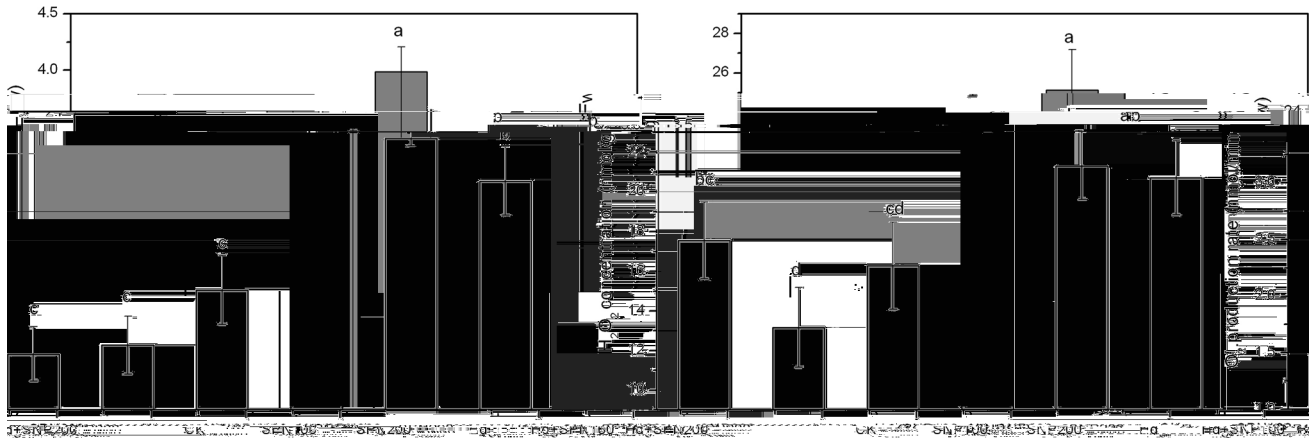

Fig. 4 Effects of NO (SNP 100 μM or 200 μM) on $O_2\cdot^-$ production rates and H_2O_2 levels in leaves of rice seedlings cultured hydroponically with or without 100 μM $HgCl_2$ treatment for 3 days. Data are presented as the mean ± SD. Values with different lowercase mean the significant difference at $P < 0.05$ level

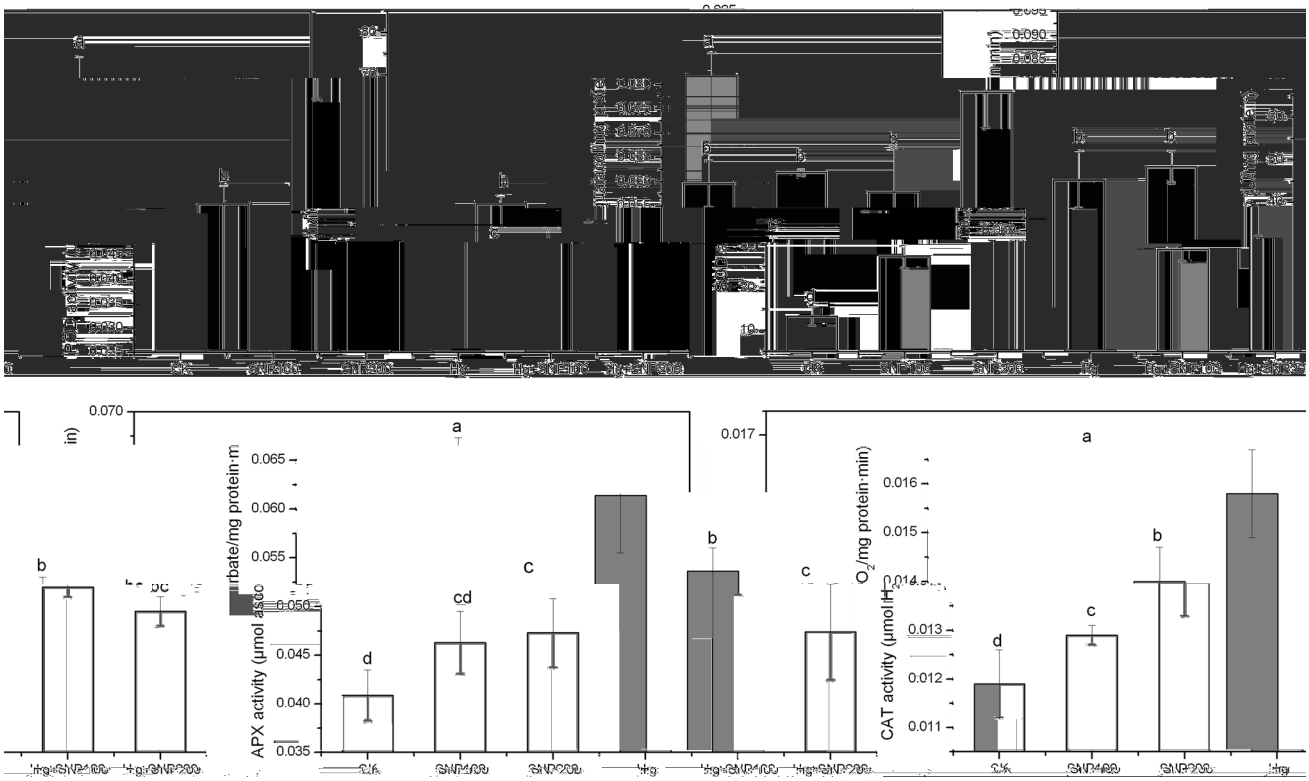

Fig. 5 Activities analysis of antioxidant enzymes, including SOD, POD, CAT, and APX, in leaves of rice seedlings cultured hydroponically under different concentrations of Hg or Hg + SNP treatments. Hg, $HgCl_2$ 100 μM; SNP100, SNP 100 μM; SNP200, SNP 200 μM. Data are presented as the mean ± SD. Values with different lowercase mean the significant difference at $P < 0.05$ level

(Kopyra and Gwóźdź 2003; Singh et al. 2009), then resulting in the enhanced tolerance.

Effects of NO and antioxidant enzyme inhibitors on growth of rice plantlets

In order to verify whether the protection of NO against oxidative damage was brought by direct quenching of peroxy radicals, inhibitors of antioxidant enzymes were exploited. DDC is an inhibitor of Cu/Zn-SOD and 0.3–3 mM DDC all inhibited the growth of rice plantlets (data not shown). As shown in Fig. 6, 3 mM DDC aggravated the toxicity of Hg. However when 200 μM SNP was added, the inhibition caused by Hg and DDC was attenuated significantly. The average shoot length was 1.15 cm, 3.8-fold to the length of 0.3 cm under the treatment of Hg + DDC. AT, is an inhibitor of catalase and 0.5–5 μM had no significant effect on growth of rice plantlets (data not shown). However, the growth was strongly inhibited by Hg + AT treatment. The shoot length of 7-day-old seedlings was only 2.45 cm, just 27.5 % in comparison with control (8.9 cm). Upon SNP application, the shoot length recovered to the length of 6.52 cm. All these data confirmed NO could prevent oxidative stress by quenching ROS directly.

Discussion

Hg is a non-essential but highly toxic heavy metal. It is easily taken by plants, accumulates in the different parts of plants, and then threatens crop yield and food safety all over the world. So it is urgent to find some effective, safety and economical measures to alleviate the phytotoxicity induced by heavy metals. Researches have proved that salicylic acid and carbon monoxide could mitigate Hg toxicity (Zhou et al. 2009; Meng et al. 2011). And the detoxifications of NO on other heavy metals, such as As, Cd, Al and Ni, have also been elucidated by researchers (Singh et al. 2009; Arasimowicz-Jelonek et al. 2011; He et al. 2012; Saxena and Shekhawat 2013). This experiment exhibited that root growth inhibition and oxidative stress of rice plant induced by excessive Hg were effectively weakened by application of NO, released from SNP (Figs. 1, 2, 4 and 5). While the other compositions released from SNP, NO_2^- and $Fe(CN)_6^{4-}$, had no ameliorative effects on inhibition induced by heavy metal (Fig. 1), which was consistent with the results reported by Xiong et al. (2009a, b) and He et al. (2012).

Root growth, including primary root elongation, lateral root development or adventitious root formation, have been

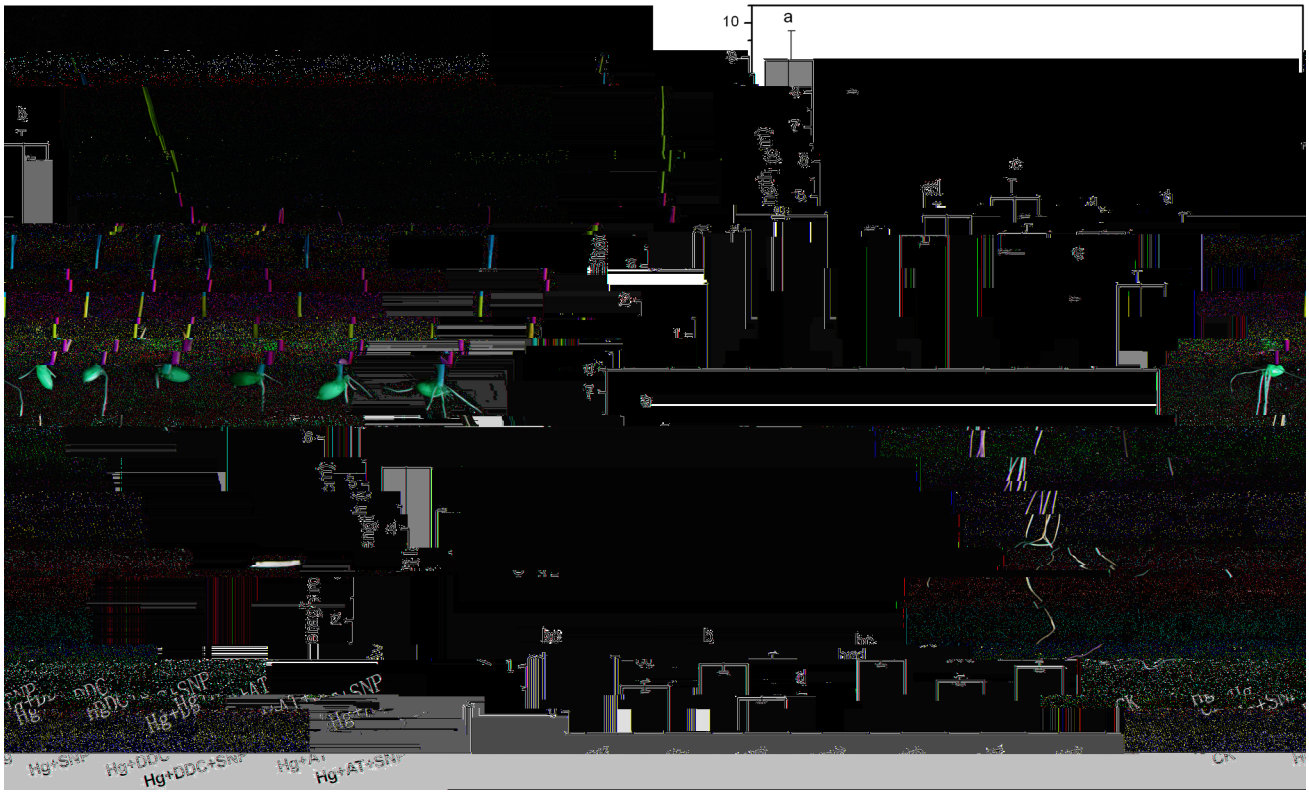

Fig. 6 Effects of NO and antioxidant enzyme inhibitors on growth of rice plantlets after seeds germination under Hg stress by agar culturing in sterilized bottles. CK, treatment without any chemical reagents; Hg, Hg Cl_2 60 μM; Hg + SNP, $HgCl_2$ 60 μM + SNP 200 μM; Hg + DDC, $HgCl_2$ 60 μM + DDC 3 mM; Hg + DDC + SNP, $HgCl_2$ 60 μM + DDC 3 mM + SNP 200 μM; Hg + AT, Hg 60 μM + AT 2 μM; Hg + AT + SNP, Hg 60 μM + AT 2 μM + SNP 200 μM. Data are presented as the mean ± SD. Values with different lowercase mean the significant difference at $P < 0.05$ level

proved to be regulated by the interaction of NO and auxin (Guo et al. 2008; Fernández-Marcos et al. 2012, He et al. 2012). Auxin stimulated lateral root formation by activating pericycle cell division (Guo et al. 2008) and is an indispensable hormone to root growth. And authors postulated NO was an important molecule operating downstream of auxin through a linear signaling pathway during root growth and development. It might be mediated by cyclic GMP (Pagnussat et al. 2003), by calcium and calcium-dependent protein kinases (Lanteri et al. 2006) or by tyrosine nitration of proteins (Yadav et al. 2013). On the other hand, since the endogenous hormone auxin is essential for root growth and development, which demonstrated by crown rootless mutant (Inukai et al. 2005), whether NO participates in the synthesis of auxin is worth to further researches. A transient increase of NO could be measured in cucumber explants after 24 h of treatment with IAA and remained at detectable level until 96 h (Pagnussat et al. 2002). He et al. (2012) also demonstrated that application of SNP resulted in the significantly increase of IAA content in wheat under Al stress. So they suggested the promoting effect of SNP on root elongation was related to the amount of endogenous IAA. In contrast, the specific NO scavenger, cPTIO [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide], delayed adventitious root emergency and significantly reduced the root length and number of the IAA-treated explants (Pagnussat et al. 2002). These data strongly indicated a role of endogenous NO in IAA-mediated root organogenesis. So scholars hypothesized NO donors were able to mimic the effect of the auxin IAA on inducing de novo root organogenesis. Nevertheless, the precise linkage of IAA → NO → rooting still cannot be distinguished. Meaningfully, a new proof was found in this study. TIBA inhibited the polar transport of IAA and aggravated the toxicity of $HgCl_2$. While SNP had an antagonistic effect to TIBA, and the root length was significantly increased (Fig. 1). These indicated that NO might promote the transport of IAA, thereby changing the morphogenesis of root.

Hg was readily taken up by plants roots and mainly accumulated in roots. Only a small proportion could be transported to shoots (Fig. 3). It was also found in earlier researches (Chen et al. 2012). NO decreased the absorption and accumulation in roots and shoots of Hg in rice (Fig. 3). On the opposite, NO could increase Cd-import

into root cells (Xiong et al. 2009a) and alleviated Cd toxicity by increasing pectin and hemicellulose contents to fix the Cd. The difference may dependent on type, time or dose of metal supplied and plant species assessed (hyperaccumulator plant or metal-sensitive plant). Hg-uptake into root cells is possibly through Fe, Cu, or Zn transporters/channels and Hg has a high ability to displace essential elements. And Hg ion is also easy to interact with anionic compounds to form insoluble precipitates (Chen and Yang 2012). So whether could NO provide negative charge or modulate ion channels, even other strategies, thereby blocking the absorption of Hg ion? These are challenging questions and need precise experiments. Nevertheless, lower deposition of Hg in root parts and highly transport of endogenous IAA caused by exogenous NO in this study provide a reasonable evidence for the role of NO in promoting the root growth under Hg stress.

Reports showed that the resistance mechanism of plant to Hg stress mostly focused on the antioxidant system. Hg

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