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Protective roles of nitric oxide on seed germination and seedling growth of rice (*Oryza sativa* L.) under cadmium stress

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ABSTRACT

Nitric oxide (NO) is a bioactive molecule in plants which mediates a variety of physiological processes and responses to biotic and abiotic stresses including heavy metals. In the present study, the effects of exogenous NO donor sodium nitroprusside (SNP) on rice seed germination and seedlings growth were investigated under Cd stress and a possible mechanism was postulated. The results indicated that 100 μM Cd significantly decreased rice seed germination index, vigor index, root and shoot lengths as well as fresh weight compared to control. Exogenous SNP dose-dependently attenuated the inhibition of rice seed germination and thereafter seedling growth caused by Cd. The promoting effect was most pronounced at 30 μM SNP. Cd exposure caused oxidative stress by elevating hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) contents in root and shoot of rice seedlings. 30 μM SNP counteracted partly Cd toxicity by reducing the H_2O_2 and MDA contents of Cd-exposed seedlings. Meanwhile, application of SNP markedly stimulated the activities of superoxide dismutases (SOD), ascorbate peroxidases (APX), guaiacol peroxidase (POD) and catalases (CAT) compared with Cd treatment alone, thereby indicating the enhancement of the antioxidative capacity in the root and shoot under Cd stress. In addition, addition of 30 μM SNP increased accumulation of proline in both root and shoot. The Cd accumulation in seedlings was significant reduced by SNP, implicating that the protective role of SNP was responsible for preventing Cd accumulation. However, the effects of SNP were reverted by addition of cPTIO, a NO scavenger, suggesting the protective roles of SNP might be related to the induction of NO. Furthermore, $K_3Fe(CN)_6$ and NO_3^-/NO_2^- had no similar roles as SNP. Based on these results, it can be concluded that SNP exerted an advantageous effect on alleviating the inhibitory effect of Cd on rice seed germination and seedling growth, which might interact with NO.

1. Introduction

Among the major environmental pollutants, cadmium (Cd) is one of the most phytotoxic heavy metals. It is released into air, water and soil through human agricultural, industrial or urban activities; notably, the high values of Cd observed in many agricultural fields are due to long-term uses of phosphate fertilizers, sewage sludge applications and waste water irrigation (He et al., 2009; Uraguchi and Fujiwara, 2012). This non-essential element is highly mobile in the soil–plant system which allows its easy entry into vegetables and can impair several vital processes, result in poor growth and low economic yield of plants, and cause toxic effects on human health through the food chain (Sanita di Toppi and Gabbrielli, 1999; Ekmekci et al., 2008; Shamsi et al., 2008). Cd-induced phytotoxicity or plant tolerant response is a complex phenomenon involving developmental changes as well as physiological and biochemical mechanisms (Sanita di Toppi and Gabbrielli, 1999; Sharma and Dietz, 2009). To counteract the toxicity of Cd, plants have developed various strategies for exudation of organic acid, retention of Cd in roots and immobilization in the cell wall (He et al., 2009). In addition, studies on different plant species have revealed that the antioxidant defense mechanism played an important role in plants under heavy metal stress (Nouairi et al., 2009; Sharma and Dietz, 2009).

In general, germination and the early seedling growth stages are key steps of the plant life and are more sensitive to metal toxicity since some of the defense mechanisms have not yet fully developed (Sanita di Toppi and Gabbrielli, 1999; Munzuroglu and Geckil, 2002; Rahoui et al., 2008; Bousbih-Sfaxi et al., 2011). Previous experiments indicated that Cd can inhibit significantly germination and the growth of *Triticum* and *Cucumis*, faba bean and pea (Munzuroglu and Geckil, 2002; Mihoub et al., 2005; Rahoui et al., 2008). This may be correlated with several disturbances in the chain of events of germinative metabolism

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which is a highly complex multistage process. One of the underlying metabolic activities following imbibition of seed is oxdative stress (Mihoub et al., 2005).

Nitric oxide (NO), a small, water and lipid soluble gas, has emerged as a major signaling molecule (Wendehenne et al., 2004). NO has been implicated in a number of diverse physiological processes in plants, including seed germination, stomatal closure, maturation and senescence, and programmed cell death responses to biotic and abiotic stresses (Beligni and Lamattina, 2000; Wendehenne et al., 2004; Singh et al., 2008; Besson-Bard et al., 2009; Xiong et al., 2010). The protective role is based on its ability to regulate the level and toxicity of reactive oxygen species (ROS). Application of exogenous NO in the form of sodium nitroprusside (SNP), as a NO donor, alleviates the adverse effects caused by heat, salt and drought stress by decreasing hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) contents and modulating the activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and guaiacol peroxidase (GPX) (Wendehenne et al., 2004; Xiong et al., 2010).

Recently, there is evidence to support a role for exogenous NO in alleviating heavy metal toxicity in plants. For example, pretreatment with SNP ameliorated toxic effects of Cd on yellow lupins, rice, sunflowers and wheat by improving the antioxidant system (Kopyra and Gwóźdź, 2003; Laspina et al., 2005; Singh et al., 2008). Under Cu stress conditions, addition of NO enhanced the antioxidative capacity and prevented the Cu induced increase in MDA content of rice and wheat seeds and scavenged the excess ROS (Hu et al., 2007). Also, treatment with NO or its donor can enhance germination and/or break dormancy in seeds (Beligni and Lamattina, 2000; Kopyra and Gwóźdź, 2003; Bethke et al., 2007). However, whether NO can stimulate germination of rice seeds under Cd stress and whether the antioxidative mechanism is involved are not yet well known. In the present work, we try to address how exogenous NO affects seed germination, seedling growth, Cd accumulation and the activities of antioxidative enzyme during rice seed germination under Cd stress, thereby providing evidences of the involvement of NO in alleviating oxidative stress during rice seeds germination.

2. Materials and methods

2.1. Growth and seeds treatment

Preliminary experiment has shown that the concentration of Cd that will result in 50 percent inhibition in root elongation was 100 μM.

Uniform healthy seeds of rice (*Oryza sativa* L. cv. Jiyou no. 9) were surface-sterilized with five percent (v/v) sodium hypochlorite for 10 min, rinsed several times with distilled water. After being soaked in distilled water at 25 ± 1 °C for 24 h, seeds were evenly dispersed on two sheets of filter papers moistened with 7 ml 100 μM Cd solution plus various concentrations of SNP (0, 10, 30, 50, 100, 200, 500 μM) in Petri dishes, covered by the lid, and incubated in the dark for seven days at 28 ± 1 °C with 80 percent relative humidity in the culturing box (LRH-250-A). Controls were obtained by moistening the filter papers with 7 ml deionized water. The germination percentage, germination index, vigor index and root and shoot length were determined. It was found that 30 μM SNP was the optimal concentration in this paper. To further verify the SNP-mediated improvement of germination and seedling growth under Cd stress, seeds were treated with deionized water (CK), 30 μM SNP, 60 μM cPTIO, 30 μM $K_3Fe(CN)_6$, 30 μM NO_3^-/NO_2^-, 100 μM Cd, 100 μM Cd+30 μM SNP (Cd+SNP), 100 μM Cd+30 μM SNP+60 μM cPTIO (Cd+SNP+cPTIO), 100 μM Cd+30 μM $K_3Fe(CN)_6$ (Cd+$K_3Fe(CN)_6$), 100 μM Cd+30 μM NO_3^-/NO_2^- (Cd+NO_3^-/NO_2^-). In this experiment, cPTIO was used as a specific NO scavenger, and $K_3Fe(CN)_6$ and NO_3^-/NO_2^- as the controls of SNP decomposition. All treatments were replicated three times. Each replicate was about at least 150 rice seeds. There were at least 50 seeds in each Petri dish. The test solutions were renewed every day to avoid any change in the concentration.

2.2. Determination of germination parameters and cadmium concentration

Seeds were considered to have germinated when both the plumule and radicle were over 2 mm long. Cumulative germination percentage (%) was recorded every day for seven days. Germination percentage, germination index and vigor index were calculated according to He et al. (2010). At seven days after different treatments, shoot length was measured from the base of the culm to the tip of the longest leaf and root length was measured from the root–shoot junction to the tip of the longest root in germinating rice seeds using ten random samples in triplicate. Prior to the fresh weight (FW) measurement, the seedlings were washed with deionized water, followed by proper blotting between filter papers.

At the end of the experiment, ten seedlings were selected at random from each Petri dish for Cd analysis. For Cd determination, the seedlings were desorbed in 20 mM Na_2-EDTA for 15 min to remove apoplastic Cd and washed thoroughly five times with distilled water. They were then oven dried at 70 °C for 72 h, weighed and digested with a $HNO_3/HClO_4$ mixture (4:1, v/v) (He et al., 2010). Cd concentration was determined by furnace atomic-absorption spectrometry (SolAAR-M6, USA). A blank digest was carried out in the same way. All measurements were performed in three replicates. Quality assurance was carried out by the determination of total Cd contents in certified reference materials (GBW 07310).

2.3. Proline determination

Proline content was measured according to the method described by Bates et al. (1973). Fresh sample was ground in 3 percent (w/v) aqueous sulphosalicylic acid. The supernatant (2 ml) was added to 2 ml of glacial acetic acid plus 2 ml of 2.5 percent (w/v) acidic ninhydrin, and then heated at 100 °C for 25 min. After the liquid was cooled, it was added to 4 ml toluene. The absorbance of the extract was read at 520 nm. The proline concentration was determined after the realization of a standard curve and expressed in mmol/g fresh weight.

2.4. Determination of lipid peroxidation and hydrogen peroxide

Accumulation of lipid peroxides in tissues was determined by estimation of malondialdehyde (MDA) content as described by Du and Bramlage (1992). Sample was homogenized in 3 ml five percent trichloracetic acid (TCA). The homogenate was centrifuged at 12,000*g* for 25 min. The 2 ml supernatant was mixed with 2 ml twenty percent TCA containing 0.5 percent thiobarbituric acid (TBA). The mixture was heated in boiling water bath for 30 min, cooled rapidly in ice bath, and centrifuged at 10,000*g* for 5 min. The absorbance of the supernatant was measured at 532 nm and the value for nonspecific absorbance at 600 nm was subtracted. The concentration of MDA was calculated by using extinction coefficient of 155 $mM^{-1}\ cm^{-1}$.

Hydrogen peroxide (H_2O_2) content was determined according to Zheng et al. (2009). The assay was based on the absorbance change of the titanium peroxide complex at 415 nm. Absorbance values were quantified using standard curve generated from known concentrations of H_2O_2.

2.5. Antioxidative enzyme assays

For the extraction of enzymes, sample was ground to powder in liquid nitrogen and homogenized in a pre-chilled mortar and pestle in 1.5 ml ice-cold extraction buffer containing 50 mM Na-phosphate buffer (pH 7.8), 1 mM ethylene diaminetetraacetic acid (EDTA), and 1.0 percent (w/v) polyvinyl-pyrrolidone (PVP). The homogenate was centrifuged at 15,000*g* for 20 min at 4 °C. The supernatant was used as the crude extract for assay of enzyme activities.

Superoxide dismutase (SOD) activity was assayed by measuring its capacity of reduction of nitro-blue tetrazolium (NBT). The absorbance of reaction solution was measured at 560 nm. One unit of SOD was defined as the enzyme activity that inhibited the reduction of nitroblue tetrazolium to blue formazan by 50 percent. Total SOD activity was expressed as U/g FW.

Activities of guaiacol peroxidase (GPX) and catalase (CAT) were determined using the method of Cakmak and Horst (1991). The activity of GPX was assayed by the change of absorbance at 470 nm due to oxidation of guaiacol. The activity of CAT was measured as a decline in the absorbance at 240 nm by H_2O_2 decomposition. The ascorbate peroxidase (APX) activity was assayed by the decrease of ascorbate absorbance at 290 nm according to Nakano and Asada (1981) by monitoring the decrease in ascorbic acid absorbance at 290 nm. A 0.01 increment of A_{470} in 1 min and a 0.1 reduction of A_{240} and A_{290} in 1 min were considered as one unit of enzyme activity of GPX, CAT and APX (U), respectively, and the activity were expressed as U/g FW.

2.6. Statistics

Data obtained were subjected to analysis of variance using SPSS statistical package version 16.0. Treatment means were compared using the least significant difference at one percent and five percent probability level. In addition, results of the different treatments were evaluated by principal component analysis (PCA) with the SPSS 16.0 software. Plots were generated using principal components (PC) 1 and 2 as axes.