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CADMIUM STRESS STIMULATES NITRIC OXIDE PRODUCTION BY WHEAT ROOTS

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Abstract

Study was conducted to elucidate the nitrite-dependent nitric oxide (NO) production by wheat roots grown in hydroponics under cadmium (Cd) stress. In a long-term Cd exposure experiment, plants were grown for 4 weeks under 1 µM Cd. The root Cd concentration in the Cd-stressed plants was 2.5 mM compared to 0.1 mM in the untreated plants. Most of the Cd taken up by plants was restricted to roots where its concentration was 9 times higher than that in shoots. Despite the high Cd concentration in roots and shoots, the plant growth was not affected. However, Cd stress caused a 1.7-fold decrease in the root respiration, whereas it produced a 2.4-fold increase in NO emission (detected by gas phase chemiluminescence). In a short-term Cd exposure experiment, freshly harvested Cd-free roots were exposed to 10 µM Cd for 3 h. Here also, the root respiration decreased by 42% and NO production increased by 73%, thus confirming the stimulatory effect of Cd stress on NO production by wheat roots.

Introduction

Nitric oxide (NO) is a versatile signal molecule synthesized both in animals and plants, and is known to participate in various complex biological functions (Schmidt & Walters, 1994; Furchgott, 1995; Durner & Klessig, 1999; Kopyra & Gwóźdź, 2004). Besides its involvement in biotic and abiotic stresses, NO is known to play a role in plant growth regulation (Beligni & Lamattina, 2000; Pagnussat *et al*., 2002; Kopyra & Gwóźdź, 2003, 2004). Most of the studies on the role of NO in plants have been focussed on the plant response to biotic stress (Van Camp *et al*., 1998; Leshem, 2000). Recent studies have shown NO as a key signal molecule modulating the expression of genes and proteins involved in the programmed cell death and plant-pathogen responses (Lamotte *et al*., 2004; Durner *et al*., 1998; Klessig *et al*., 2000). During plant-pathogen interactions, NO can alleviate the negative effects of reactive oxygen species (ROS) eg., cell death, ion leakage or DNA fragmentation (Beligni & Lamattina, 1999). Recently, NO has been shown to also alleviate the harmful effects of abiotic stresses like drought, salinity, heat and mechanical injury (Lamattina *et al*., 2003; Liu *et al*., 2007).

Relatively few studies deal with the role of NO in plants under heavy metal stress. Cadmium (Cd) is amongst the most toxic heavy metals for human and plants entering into the environment mainly through phosphate fertilizers and industrial waste disposal (Das *et al*., 1997; Yilmaz *et al*., 2006). In plants, Cd toxicity causes growth inhibition by affecting metabolic processes, particularly photosynthesis and respiration (Stroiński, 1999; Ferreira *et al*., 2002; Kopyra & Gwóźdź, 2003). There are reports on the involvement of exogenous NO in protecting plants against oxidative stress induced by

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heavy metals (Hsu & Kao, 2004; Wang & Zang, 2005; Kopyra *et al*., 2006). If the protective role of NO is through scavenging ROS, the endogenous NO production should decrease under heavy metal stress. However, the effect of heavy metals like Cd on the endogenous NO production by plants has not been reported. The present study demonstrates the trends in NO_2^--dependant NO production by wheat roots grown hydroponically under Cd stress.

Materials and Methods

Wheat (*Triticum aestivum* L.) variety 'Inqalab-91' was selected for the present study. In a preliminary experiment in hydroponics, this variety was found fairly resistant to Cd stress without showing Cd toxicity symptoms or negative effects on the root and shoot growth after a 4-week exposure to 1-10 µM Cd (results not shown). Seeds were germinated in quartz sand and the 7-day old seedlings transplanted (6 plants pot^{-1}) to 2-L pots containing 10% nutrient solution. The nutrient solution contained: $MgSO_4.7H_2O$, 0.2 mM; KCl, 0.4 mM; $CaCl_2.2H_2O$, 0.4 mM; KNO_3, 0.3 mM; $(NH_4)_2SO_4$, 0.3 mM; KH_2PO_4, 0.2 mM; Na-Fe-EDTA, 0.126 µM; and trace elements according to Johnson *et al*. (1975); pH, 6.0. After 7 days, the plants were supplied with 100% nutrient solution either without (control) or with 1 µM Cd as $CdCl_2.H_2O$. Nutrient solution and Cd were replaced after every 48 h. After 4 weeks under treatments, plants were harvested for determination of biomass yield, Cd concentration, NO production and root respiration. Alternatively, for the short-term Cd exposure experiment, normal roots (from Cd-free plants) were incubated for 3 h under NO_2^- and then 10 µM Cd was added to the incubation medium and NO production followed for a further 3 h. After termination of the experiment, the same roots were used for the determination of root respiration.

For the determination of NO production rate in both experiments, roots were cut into segments (about 1 g fresh weight, 1 cm long) and suspended in beakers containing 10 ml of 20 mM Hepes buffer (pH 7.6) and 0.5 mM $NaNO_2$. Beakers were placed in a glass cuvette that was connected to a chemiluminescence NO analyzer (Ecophysics CLD 770 Al ppt, Munich, Germany). The vessel was placed on a rotary shaker at 130 rpm and a continuous precise stream of NO-free air (1.3 L min^{-1}) was conducted through the vessel into the analyzer. The concentration of NO in the outcoming stream was measured during 20 sec intervals, as described by Planchet *et al*. (2005). For polarographic determination of the root respiration, about 0.05 g (fresh weight) root segments were incubated in 2 ml buffer solution in a temperature-controlled Perspex cuvette (25ºC) under continuous stirring, and O_2 consumption was continuously monitored (Stoimenova *et al*., 2003).

Roots and shoots were dried at 60ºC till constant weight and ground (<1 mm) before analysis of Cd. Plant material was digested in a mixture of HNO_3:$HClO_3$ (5:1) and Cd concentration determined by flame atomic absorption spectrometry. Data were subjected to an analysis of variance followed by Duncan's multiple range test (Gomez & Gomez, 1980).

Results and Discussion

Plants grown for 4 weeks under 1 µM Cd accumulated appreciable amounts of Cd both in roots and shoots (Table 1). Relative to the control, Cd stress caused a 19-fold increase in the root Cd concentration, whereas the corresponding increase in the shoots was 11-fold (p<0.05). Most of the Cd taken up by plants was restricted to roots where its concentration was 9 and 15 times higher than that of shoots under control and 1 µM Cd treatment, respectively. These results are consistent with those of earlier studies with

graminaceous plants showing restriction of Cd mainly in the roots and a limited transport to the shoot component (Hatch, *et al*., 1988; Liu *et al*., 2006). Despite the high Cd concentration in roots and shoots under Cd stress, the biomass yield was not affected. However, roots of plants grown under Cd stress respired at a significantly lower rate than those of untreated plants ($p<0.05$). In contrast to expectations, a 2.4-fold increase in NO production over controls was recorded for roots grown under Cd stress.

In the short-term Cd exposure experiment, immediately after the addition of 10 µM Cd, NO emission increased continuously and was not at steady state even after 3 h (Fig. 1). Before Cd addition, NO production was 41.6 n mole g^{-1} FW h^{-1}; and 3 h after addition of 10 µM Cd, the NO emission rate significantly increased to 71.8 n mole g^{-1} FW h^{-1} (Fig. 1, Table 2). The short-term Cd exposure also decreased the root respiration by 42%, which coincided with a 15-fold increase in the root Cd concentration. In order to check whether NO production by roots was to some extent non-enzymatic, KCN (2 mM) was added to the root segments in order to inhibit the nitrate reductase and the mitochondrial electron transport, which are the major sources of nitrite-dependent NO in roots. In this case, NO emission was completely abolished indicating that the non-enzymatic NO production from nitrite was negligible.

Table 1. Effect of long-term (4-week) Cd exposure of wheat plants on the biomass yield, Cd concentration, root respiration and NO_2^--dependent NO production by roots.

Parameter	Treatment	
	Control	1 µM Cd
Shoot dry weight (g $plant^{-1}$)[a]	0.87 ± 0.2 a[b] (4.15 ± 1.22 a)	1.04 ± 0.18 a (5.21 ±0.94 a)
Shoot Cd concentration (mM)[c]	0.015 ± 0.009 b (1.36 ± 0.85 b)	0.168 ± 0.007 a (15.15 ± 0.64 a)
Root dry weight (g $plant^{-1}$)[a]	0.076 ± 0.016 a (1.40 ± 0.35 a)	0.105 ± 0.023 a (1.88 ± 0.66 a)
Root Cd concentration (mM)[c]	0.13 ± 0.04 b (14.09 ± 3.97 b)	2.53 ± 0.16 a (267.84 ± 16.61 a)
Root NO production (nmole NO g^{-1} FW h^{-1})	18.27 ± 4.76 b	44.27 ± 1.52 a
Root respiration (µmole O_2 g^{-1} FW h^{-1})	50.17 ± 3.36 a	29.35 ± 4.20 b

[a]Figures in parentheses represent fresh weight (g $plant^{-1}$).
[b]Mean ± SD (n=3); figures in a row followed by different letter are significantly different at $p<0.05$ (Duncan's multiple range test).
[c]Cd concentration on basis of tissue water content; figures in parentheses represent Cd concentration (µg g^{-1}) on dry weight basis.

Table 2. Effect of short-term (3-hour) Cd exposure of wheat root segments on Cd concentration, root respiration, and NO_2^--dependent NO production.

Parameter	Before Cd addition	3 h after 10 µM Cd addition
Cd concentration (mM)[a]	0.11 ± 0.01 b[b] (11.90 ± 1.42 b)	1.67 ± 0.22 a (177.63 ± 22.23 a)
Root Respiration (µmole O_2 g^{-1} FW h^{-1})	18.6 ± 1.14 a	10.73 ± 1.33 b
NO production (nmole NO g^{-1} FW h^{-1})	41.58 ± 1.90 b	71.84 ± 9.14 a

[a]Cd concentration on basis of tissue water content; figures in parentheses represent Cd concentration (µg g^{-1}) on dry weight basis.
[b]Mean ± SD (n=3); figures in a row followed by different letter are significantly different at $p<0.05$ (Duncan's multiple range test).

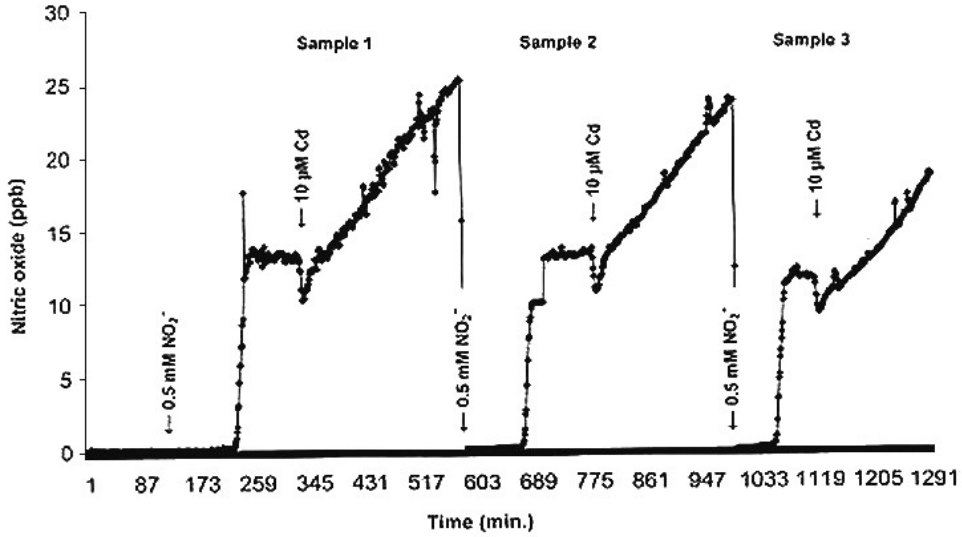

Fig. 1. Time course of NO_2^--dependent NO production by wheat root segments before and during 3-h exposure to 10 µM Cd.

The high Cd concentration in wheat roots and shoots grown on Cd, and the lack of plant growth inhibition indicate that some acclimation mechanisms prevail in the wheat cultivar 'Inqalab-91' conferring resistance against Cd stress. Cadmium is known to promote the generation of ROS like superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2) excessive levels of which cause oxidative damage of biomolecules like lipids, proteins and nucleic acids (Yamamoto *et al*., 2002; Sanità di Toppi & Gabbrielli, 1999). The role of antioxidant defense molecules like ascorbate, glutathione and α-tocopherol in the detoxification of ROS produced under biotic and abiotic stress is well documented (Hess, 1993; Winkler *et al*., 1994; Grene, 2002). A crucial plant response to cope with the Cd-induced oxidative stress is the upregulation of antioxidant enzymes like catalase, superoxide dismutase and glutathione reductase (Pereira *et al*., 2002). There have been recent reports on the involvement of exogenous NO in protecting plants against oxidative stress induced by heavy metals. Exogenous application of NO to Cd-treated soybean cell suspension showed an antioxidative role of NO through direct scavenging of ROS and stimulation of the antioxidant system (Kopyra *et al*., 2006). A similar protective role of exogenous NO in Al-induced oxidative stress has been reported for roots of *Cassia tora* (Wang & Zang, 2005). In these studies Sodium nitroprusside (SNP) was used as NO donor and the NO production was measured by NO-specific fluorochrome diaminofluorescein diacetate (DAF-2DA). However, the latter has been shown to react not directly with NO, but most probably with some oxidation product, eventually N_2O_3 (Planchet & Kaiser, 2006) and gives only semi-quantitative information on NO production. Cadmium toxicity in rice leaves was also reduced by the application of exogenous NO donors like N-tert-butyl-α-phenylnitrone, 3-morpholinosydonimine and SNP that probably scavenge ROS (Hsu & Kao, 2004). If the protective effects of NO would be due to a reaction of the NO radical with ROS that can be produced under heavy metal stress (Yamamoto *et al*., 2002; Sanitàa di Toppi & Gabbrielli, 1999), a prediction would be that Cd stress should actually decrease NO

emission from plant tissues. However, the present study demonstrates that the opposite is true i.e., NO_2^--dependent NO production by wheat roots is increased under Cd stress. In the present study, it remained unclear whether NO production was actually increased to such a high extent that more NO was emitted in spite of a partial oxidation of NO through ROS. It is also possible that the observed stimulation of NO production was an indirect effect of Cd, i.e. through inhibition of root respiration; the decreased ATP level in roots is known to stimulate the nitrate reductase activity (Glaab & Kaiser 1993) thus the NO_2^--dependent NO production. Detailed studies are needed to elucidate the mechanisms behind the stimulatory effect of Cd on NO production by roots.

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