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Nitric oxide production induced by heavy metals in *Brassica juncea* L. Czern. and *Pisum sativum* L.

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ABSTRACT In plants, nitric oxide (NO) has multiple roles in defence reactions under abiotic stresses, including heavy metal load. Literature data suggest that there is a causal relationship between NO and iron metabolism but the effects of essential micronutrients/toxic heavy metals on NO production have not been investigated. In this study our aim is to demonstrate the possible role of NO in the plant response to heavy metals in the metal accumulator *Brassica juncea* and the crop plant *Pisum sativum* grown in the presence of either 100 μM cadmium, copper or zinc. NO production was measured in the root tips with fluorescent method, using 4,5-diaminofluorescein diacetate (DAF-2 DA), a specific dye to nitric oxide. We obtained different NO levels with the different heavy metal load: the most effective metal were copper and cadmium, in this case the NO production became double after one week treatment. In case of copper load, two-phase kinetics was found: a fast NO burst in the first six hours was followed by a slower, gradual increase. The fast appearance of NO in the presence of cupric ions suggest that it can be a novel reaction hitherto not studied in plants under heavy metal stress. After long-term treatment, NO levels were inversely related to the nitrite concentrations originated from nitrate reductase activity suggesting the conversion of nitrite to nitric oxide by the known enzymatic ways.

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KEY WORDS

nitric oxide
copper-induced NO burst
heavy metal stress
Pisum sativum
Brassica juncea

Nitric oxide is an uncharged lipophilic gas. It can exist as three interchangeable forms: the radical ($NO^{\cdot}$); nitrosonium cation (NO^{+}); and nitroxyl anion (NO^{-}). Compared with other free radicals it has a relatively long biological half-life. At low concentration (less than 1 μmol/l) minutes to hours, at higher concentrations in the order of seconds.

The various effects of this molecule in animal organisms (blood pressure regulation, antioxidant effect, cell death, DNA damage) is well known and thoroughly investigated. Also, in plants, its far-reaching roles were pointed out for example in growth inhibition, stimulation of secondary root formation, inhibition of photosynthesis, programmed cell death and abiotic stress responses.

In plants, NO can be generated via enzymatic and non-enzymatic pathways. The enzymatic pathways are catalysed by cytosolic nitrite reductase (cNR), NO synthase (NOS) or NOS-like enzymes and nitrite:NO reductase (Ni-NOR), respectively. Non-enzymatic way is the nitrite dismutation to NO and nitrate at acidic pH values (Stöhr et al. 2002; Neill et al. 2003; Graziano and Lamattina 2005).

The generated NO can induce various effects, or react with reactive oxygen species like superoxide and hydrogen peroxide to generate peroxynitrite. NO can also react with other potential signalling molecules, which are likely to be produced temporally and spatially alongside NO.

The quantification of endogenous NO is not easy because the sampling and instrumentation like gas chromatography and mass spectrometry. Instead, the use of fluorescent methods is an excellent solution for *in situ* and *in vivo* detection of NO with fluorescent microscopy and NO-specific dyes. In this study, the time dependence of NO-production is investigated as stress response to heavy metals in roots.

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Materials and Methods

Plant materials

Plants were grown in Hoagland nutrient solution under controlled conditions. At the age of 4 weeks (*Brassica*) or 1 week (*Pisum*), 100 μM Cd, Cu or Zn were added to the nutrient solution for 5-day period. Time dependence experiments were carried out with 2-week old plants. Root samples were stained for NO with diamino-fluorescein diacetate (DAF-2 DA; Pedroso et al. 2000). As control, untreated plants were used, while for positive control, roots were treated with 1 mM sodium nitroprusside (SNP) as NO-generator for 5 hours before sectioning and staining with DAF-2 DA.

Fluorescent microscopy

The indicator is the 4,5-diaminofluorescein diacetate (DAF-2 DA), which is a specific membrane permeable dye to NO. Inside the cell it can react with intracellular esterases forming 4,5-diaminofluoresceine which can react with nitric oxide. The result is a fluorescent heterocycle form, which is non-permeable. We detect the green fluorescence with Zeiss Axiowert 200M fluorescent microscope equipped with Ax-

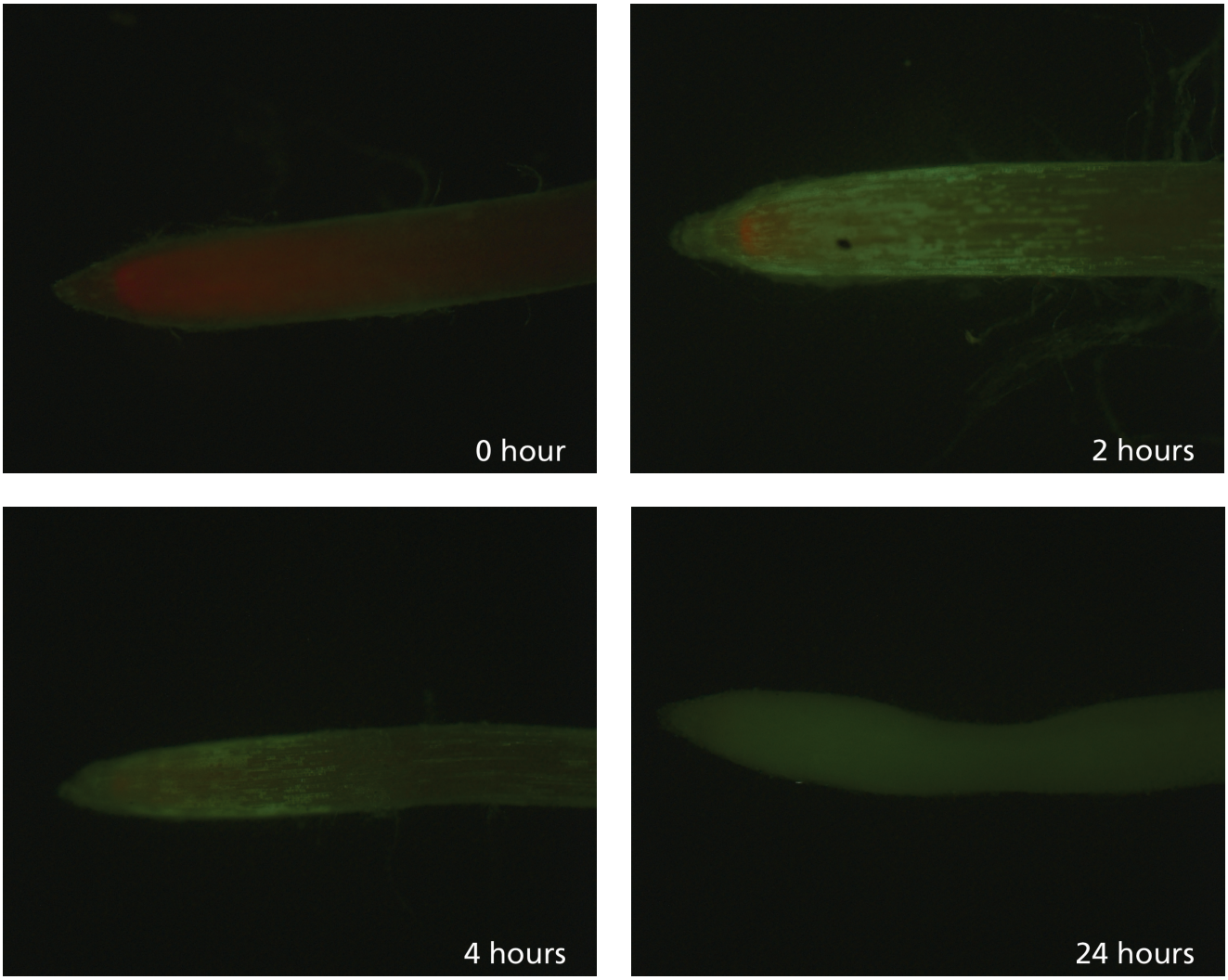

Figure 1. Time dependence of NO production in Pisum root after 0, 2, 4 and 24 hours. Root samples were stained for NO with diamino-fluorescein diacetate (DAF-2 DA) and were investigated under fluorescence microscope type Zeiss Axiovert 200M equipped with Axiovision 4.4 software programme.

iovision 4.4 software programme and with high resolution, sensitive colour digital camera and Axiovision 4.2 software from Zeiss Inc.

Measurement of nitrate reductase (NR) activity

NR activity was measured as described in Kolbert et. al. (2005).

Results

Time-dependence of heavy metal induced NO production

NO production was measured after 100 μM heavy metal treatment in both plant species. The measurements were made after 1, 2, 4, 6, 12, 24 and 48 hours.

On the basis of the time course of metal-induced NO production, Cu clearly showed a biphasic reaction (Fig. 1), namely, a fast burst of NO release followed by a slow increase, while Cd and Zn induced only the slow response. In case of Cu treatment, the nitric-oxide level was 7-8 times higher in the first 3 hours then in case of Zn and Cd in both plants (Fig. 2). Probably this nitric-oxide burst originate from non-enzymatic reactions (lipid peroxidation or the production of reactive oxygen species). Cadmium and zinc did not initiate this fast response. After 24 hours a slow response begun – probably on enzymatic background.

After the long-lasting treatment (5 days), Zn-induced NO production was the lowest, probably because of the complexation or the least toxic nature of this metal, compared to Cd and Cu-induced NO levels (Fig. 3).

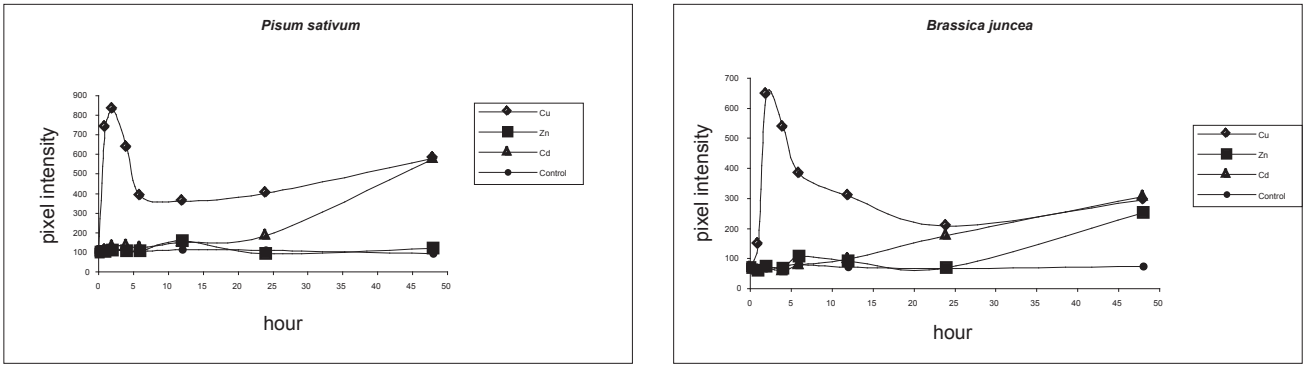

Figure 2. Time dependence of NO production in the presence of 100 μM Cu, Zn and Cd in *Pisum* and *Brassica* roots.

Effect of heavy metal stress on the *in vivo* activity of nitrate reductase

Nitrate reductase activities were measured after 24 hours in control and treated roots. The lowest NR activities coincided with the highest NO productions in Cu-treated plants. In this way, there was an inverse relationship between NR activity (measured as the quantity of the product, nitrite) and NO levels indicating that NO may originate from the enzymatically produced nitrite (Fig. 4).

These data suggest that the fast response may include the NO- and Cu^{2+}-related production of reactive oxygen species (with Cu^{2+} as catalyst), while the slow response in NO production may be due to the enzymatic side reaction of the nitrate reductase.

Discussion

The targets of toxic amounts of heavy metals are first of all, the essential metalloenzymes, which at the same time serve as antioxidant enzymes as well: superoxide dismutase (Cu/Zn, Mn, Fe, Ni), catalase (Fe-hem), glutathione peroxidase (Se, selenocysteine), peroxidase (Fe-hem), ascorbic acid oxidase (Cu). Reactive oxygen species can be generated in different reactions, but from point of view our present results the transition metal copper received importance.

Of the investigated metals, Cu was outstanding in NO production, probably through a hitherto unknown step of the signal transduction pathway. A key to the solution can be formation of NO radical from the nitroxyl anion (NO^-), a reaction catalyzed efficiently by both Cu^{2+} and Cu^+ (Nelli et al. 2000). Nitroxyl anion can be the product through the decomposition of nitrosothyols (Arnelle and Stamler 1995). Another interesting result in literature is that the copper-containing nitrite reductase common in bacteria was purified from the eukaryote *Fusarium*. The reaction product of the enzyme is mainly NO, and its activity can be restored by cupric ions (Kobayashi and Shoun 1995).

Since roots int he presence Zn and Cd did not show the fast response in NO production, it can be supposed that mechanism of NO production is differing from that of in the presence of copper. Data for NR reductase activity may indicate the NO generation by Ni-NOR and PM-NR (Stöhr and Ullrich 2002).

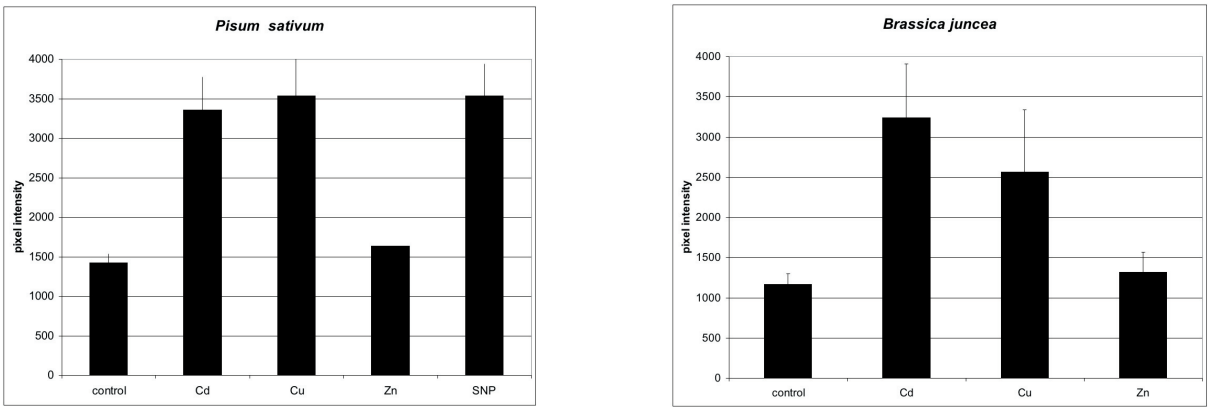

Figure 3. NO production after 5-day treatments with 100 μM Cu, Zn, Cd (and 1mM SNP in *Pisum*) in *Pisum* and *Brassica* roots.

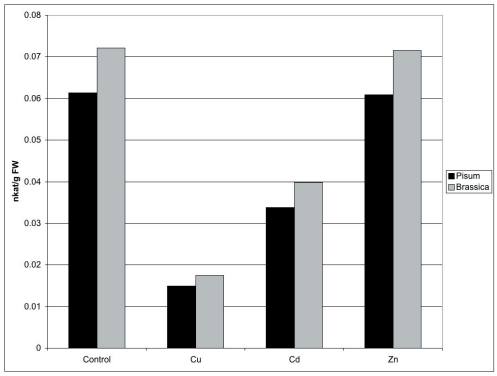

Figure 4. Effects of 100 μM heavy metal treatments on the *in vivo* NR activity.

Acknowledgements

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