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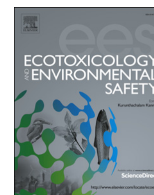

Different zinc sensitivity of *Brassica* organs is accompanied by distinct responses in protein nitration level and pattern

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ABSTRACT

Zinc is an essential microelement, but its excess exerts toxic effects in plants. Heavy metal stress can alter the metabolism of reactive oxygen (ROS) and nitrogen species (RNS) leading to oxidative and nitrosative damages; although the participation of these processes in Zn toxicity and tolerance is not yet known. Therefore this study aimed to evaluate the zinc tolerance of *Brassica* organs and the putative correspondence of it with protein nitration as a relevant marker for nitrosative stress. Both examined *Brassica* species (*B. juncea* and *B. napus*) proved to be moderate Zn accumulators; however *B. napus* accumulated more from this metal in its organs. The zinc-induced damages (growth diminution, altered morphology, necrosis, chlorosis, and the decrease of photosynthetic activity) were slighter in the shoot system of *B. napus* than in *B. juncea*. The relative zinc tolerance of *B. napus* shoot was accompanied by moderate changes of the nitration pattern. In contrast, the root system of *B. napus* suffered more severe damages (growth reduction, altered morphology, viability loss) and slighter increase in nitration level compared to *B. juncea*. Based on these, the organs of *Brassica* species reacted differentially to excess zinc, since in the shoot system modification of the nitration pattern occurred (with newly appeared nitrated protein bands), while in the roots, a general increment in the nitroproteome could be observed (the intensification of the same protein bands being present in the control samples). It can be assumed that the significant alteration of nitration pattern is coupled with enhanced zinc sensitivity of the *Brassica* shoot system and the general intensification of protein nitration in the roots is attached to relative zinc endurance.

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1. Introduction

Zinc is typically the second most abundant metal in organisms after iron (Fe) and ~9% of the eukaryote proteome contains zinc (Andreini and Bertini, 2009) suggesting its fundamental role in physiological processes. Indeed, zinc is involved in protein synthesis and in carbohydrate, nucleic acid, lipid metabolism and it is the only metal represented in all six enzyme classes (oxidoreductases, hydrolases, transferases, lyases, isomerases, and ligases) (Broadley et al., 2007). Despite its necessity, at supraoptimal concentrations zinc can explicate phytotoxic effects as well. Generally, agricultural soils contain 10–300 μg Zn g^{-1}; however the Zn content of the soils can be enhanced by natural and anthropogenic activities including mining, industrial and agricultural practices. The pollution of soil by zinc has been a major environmental concern (Zarcinas et al., 2004). In non-tolerant plants, zinc toxicity occurs above 100–300 mg/kg dry weight tissue concentration. Toxic symptoms at the whole plant level involve reduced germination rate and biomass production (Munzuroglu and Geckil, 2002), chlorosis, necrosis (Ebbs and Uchil, 2008), loss of photosynthetic activity (Shi and Cai, 2009), genotoxicity and disturbances in macro-and microelement homoeostasis (Jain et al., 2010). Excess Zn may affect photosynthesis at different sites, including, *inter alia*, photosynthetic pigments, photosynthetic electron transport, RubisCo activity (Krupa and Baszynski, 1995). At cellular level, zinc toxicity materializes through oxidative stress-associated lipid peroxidation, causing membrane destabilization in the plasmalemma, mitochondrial and photosynthetic membranes as well (Rout and Das, 2003).

The non-redox active zinc has the ability to bind tightly to oxygen, nitrogen or sulphur atoms, hereby inactivating enzymes by binding to their cysteine residues (Nieboer and Richardson, 1980). Also, zinc is able to cause secondary oxidative stress by replacing other essential metal ions in their catalytic sites (Schützendübel and Polle, 2002). During zinc-triggered oxidative

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stress, reactive oxygen species (ROS), such as superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (●OH) are commonly generated as it was revealed by several authors (e.g. Morina et al., 2010; Jain et al., 2010). The level of ROS is needed to be strictly regulated by complex mechanisms in plants (Apel and Hirt, 2004). These include several enzymes such as ascorbate peroxidase (APX, EC 1.11.1.11), glutathione reductase (GR, EC 1.6.4.2), catalase (CAT, EC 1.11.1.6) superoxide dismutase (SOD, EC 1.1.5.1.1), and non-enzymatic, soluble antioxidants such as glutathione and ascorbate, among others. The activity of several antioxidant enzymes and antioxidant contents was shown to be affected by zinc (Cuypers et al., 2002; Di Baccio et al., 2005; Tewari et al., 2008; Li et al., 2013).

Besides ROS, reactive nitrogen species (RNS) are also formed as the effect of wide variety of environmental stresses. The accumulation of these nitric oxide (NO)-related radicals and non-radical molecules (e.g. peroxynitrite, ONOO-, S-nitrosoglutathione, GSNO) leads to nitrosative stress during which one of the principle post-translational modifications is tyrosine nitration in proteins yielding 3-nitrotyrosine (Corpas et al., 2013). During this peroxinitrite-catalyzed reaction an addition of a nitro group to one of the two equivalent ortho carbons in the aromatic ring of tyrosine residues (Gow et al., 2004) takes place causing steric and electronic perturbations, which modify the tyrosine's capability to function in electron transfer reactions or to keep the proper protein conformation (van der Vliet et al., 1999). In most cases nitration results in the inhibition of the protein's function (Corpas et al., 2013). Furthermore, tyrosine nitration has the ability to influence several signal transduction pathways through the prevention of tyrosine phosphorylation (Galetskiy et al., 2011).

Although oxidative stress triggered by heavy metals is well characterized in different plant species, until today, very little is known about heavy metal-, particularly essential element excess-induced nitrosative processes such as alterations in RNS metabolism and tyrosine nitration. Therefore, the main goal of this work was to evaluate and compare the ROS-RNS metabolism and the consequent protein nitration in the root and shoot system of two economically important and moderately zinc accumulator plants (Ebbs and Kochian, 1997), Indian mustard (*Brassica juncea*) and oilseed rape (*Brassica napus*) exposed to prolonged zinc excess. Furthermore, the determination of possible correspondence between the changes in protein nitration and zinc tolerance was also a relevant issue of this study.

2. Materials and methods

2.1. Plant material and growth

Seeds of Indian mustard (*Brassica juncea* L. Czern. cv. Negro Caballo) were obtained from the Research Institute for Medicinal Plants of Budakalász, Hungary and the oilseed rape (*Brassica napus L.*) seeds from the Cereal Research Non-Profit Ltd. of Szeged, Hungary. The seeds of both species were surface-sterilized with 5% (v/v) sodium hypochlorite and then placed onto perlite-filled Eppendorf tubes floating on full-strength Hoagland solution where they grew for nine days. The nutrient solution contained 5 mM $Ca(NO_3)_2$, 5 mM KNO_3, 2 mM $MgSO_4$, 1 mM KH_2PO_4, 0.01 mM Fe-EDTA, 10 μM H_3BO_3, 1 μM $MnSO_4$, 5 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 0.1 μM $(NH_4)_6Mo_7O_{24}$ and 10 μM $AlCl_3$. The nine-day-old seedlings were treated with 50, 150 or 300 μM $ZnSO_4$ for additional fourteen days. During the whole experimental period, the control plants were kept in full strength Hoagland solution containing 5 μM $ZnSO_4$. The plants were grown in a greenhouse at a photon flux density of 150 μmol m^{-2} s^{-1} (12/12 h light/dark cycle) at a relative humidity of 55–60% and 25 ± 2 °C.

All chemicals used during the experiments were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

2.2. Element content analysis

The concentrations of microelements were measured by using inductively coupled plasma mass spectrometer (ICP-MS, Thermo Scientific XSeries II, Asheville, USA) according to Feigl et al. (2013). Root and shoot material were harvested separately and rinsed with distilled water. After the drying on 70 °C for 48 h and digestion of the plant material (digestion process: 6 ml 65% (w/v) nitric acid was added to the samples followed by 2 h of incubation; then 2 ml of 30% (w/v) hydrogen-peroxide was added then the samples were subjected to 200 °C and 1600 W for 15 min), the values of Zn and other microelement (Fe, Mn, B, Cu, Mo, and Ni) concentrations were determined. The concentrations of Zn are given in mg/g dry weight (DW), while the concentrations of other microelements are given in μg/g DW.

2.3. Measurement of photosynthetic pigment composition

In the leaves of the control and Zn-treated *Brassica* species, the amount of chlorophyll *a, b* and total carotenoids were determined according to Lichtenthaler (1987). The calculated amounts of the pigments are expressed as μg pigment/g fresh weight.

2.4. Shoot morphological measurements

The fresh weights (FW) and the dry weights (DW) of the carefully separated shoot material were measured on the 14th day of the treatment using a balance. Leaf area was determined on at least 10 specimens in every case by using a grid and ImageJ software (National Institute of Mental Health, Bethesda, Maryland, USA).

2.5. Measurement of chlorophyll fluorescence parameters

Chlorophyll fluorescence parameters were measured using a Pulse Amplitude-Modulated Fluorometer (Program "Run 8", PAM 200 Chlorophyll Fluorometer, Heinz Walz GmbH, Effeltrich, Germany). Leaves of treated and control plants were first dark adapted for 30 min and Fm, Fm', Ft and Fo' parameters were measured in the function of increasing light intensity (PAR=Photosynthetic Active Radiation) from 60 to 850 μmol photons/m/s. From these parameters the effective quantum yield of PSII ($\text{Yield}=(Fm'-Ft)/Fm'$), electron transport rate ($\text{ETR}=\text{Yield}\times\text{PAR}\times 0.5\times 0.84$), photochemical quenching ($qP=(Fm'-Ft)/(Fm'-Fo')$) and non-photochemical quenching ($NPQ=(Fm-Fm')/Fm'$) were calculated and recorded. All measurements were carried out on leaves from five different plants in three parallel experiments.

2.6. Root morphological measurements

The length of the primary root (cm) and the first six lateral roots from the root collar (cm) were determined manually. Also the visible lateral roots were counted and their number is expressed as pieces/root.

2.7. Detection of viability loss, reactive oxygen- (ROS) and nitrogen species (RNS) in the root tissues

In all cases, approx. two cm-long segments were cut from the root tips and these were incubated in 2 mL dye/buffer solutions in Petri-dishes with 2 cm diameter. After the staining procedure, the root samples were prepared on microscopic slides in buffer solution.

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