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Zinc induces distinct changes in the metabolism of reactive oxygen and nitrogen species (ROS and RNS) in the roots of two *Brassica* species with different sensitivity to zinc stress

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• **Background and Aims** Zinc (Zn) is an essential micronutrient naturally present in soils, but anthropogenic activities can lead to accumulation in the environment and resulting damage to plants. Heavy metals such as Zn can induce oxidative stress and the generation of reactive oxygen and nitrogen species (ROS and RNS), which can reduce growth and yield in crop plants. This study assesses the interplay of these two families of molecules in order to evaluate the responses in roots of two *Brassica* species under high concentrations of Zn.

• **Methods** Nine-day-old hydroponically grown *Brassica juncea* (Indian mustard) and *B. napus* (oilseed rape) seedlings were treated with $ZnSO_4$ (0, 50, 150 and 300 µM) for 7 d. Stress intensity was assessed through analyses of cell wall damage and cell viability. Biochemical and cellular techniques were used to measure key components of the metabolism of ROS and RNS including lipid peroxidation, enzymatic antioxidants, protein nitration and content of superoxide radical (O_2^-), nitric oxide (NO) and peroxynitrite (ONOO⁻).

• **Key Results** Analysis of morphological root damage and alterations of microelement homeostasis indicate that *B. juncea* is more tolerant to Zn stress than *B. napus*. ROS and RNS parameters suggest that the oxidative components are predominant compared with the nitrosative components in the root system of both species.

• **Conclusions** The results indicate a clear relationship between ROS and RNS metabolism as a mechanism of response against stress caused by an excess of Zn. The oxidative stress components seem to be more dominant than the elements of the nitrosative stress in the root system of these two *Brassica* species.

Key words: *Brassica juncea, Brassica napus,* Indian mustard, oilseed rape, excess zinc, reactive oxygen species, ROS, oxidative stress, reactive nitrogen species, RNS, nitrosative stress, protein nitration.

INTRODUCTION

Heavy metal contamination is an increasingly serious problem for the environment and for agriculture. According to the World Health Organization, 31 % of the world's population is potentially at risk of zinc (Zn) deficiency (World Health Organization, 2005); however, at the same time, Zn contamination also appears to be a increasing problem over recent decades (Zarcinas et al., 2004). The most important sources of Zn pollution in the environment are mostly anthropogenic, such as mining, waste disposal, electroplating or smelting (Bacon and Dinev, 2005; Bi et al., 2006). Being an essential micronutrient, Zn plays an important role as a cofactor in numerous enzymatic reactions involved in protein synthesis and in carbohydrate, nucleic acid and lipid metabolism (Broadley et al., 2007). On the other hand, Zn excess may have a negative effect on plants. Among others, seed germination and plant growth inhibition (Mrozek and Funicelli, 1982; Wang et al., 2009), changes in root development (Lingua et al., 2008), loss of membrane integrity (Stoyanova and Doncheva, 2002) or cell death (Chang et al. 2005) have been determined to be effects of Zn exposure. The mechanisms behind Zn toxicity are not completely

understood; competition for catalytic sites or transporters (González-Guerrero *et al.*, 2005), evidence for Zn-induced micronutrient deficiency (Bonnet *et al.*, 2000; Wang *et al.*, 2009) or induction of oxidative stress (Wintz *et al.*, 2003) has been provided.

Non-redox active heavy metals such as Zn can cause oxidative stress by blocking essential functional groups in biomolecules because of their ability to bind strongly to oxygen, nitrogen or sulphur atoms, thereby inactivating enzymes by binding to their cysteine residues (Nieboer and Richardson, 1980), or by replacing other essential metal ions in their catalytic sites (Schützendübel and Polle, 2002). During oxidative stress, reactive oxygen species (ROS), such as superoxide anion (O_2^{-}) , hydrogen peroxide (H_2O_2) and hydroxyl radicals (·OH), are commonly generated. High levels of ROS are able to damage macromolecules; thus ROS concentrations need to be strictly controlled 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) and superoxide dismutase (SOD; EC 1.1.5.1.1), and non-enzymatic,

© The Author 2014. Published by Oxford University Press on behalf of the Annals of Botany Company. All rights reserved. For Permissions, please email: journals.permissions@oup.com soluble antioxidants such as glutathione and ascorbate, among others.

In addition to ROS, the term reactive nitrogen species (RNS) is used extensively to describe the family of nitric oxide (NO)related molecules, such as peroxynitrite (ONOO⁻), dinitrogen trioxide (N₂O₃), dinitrogen tetraoxide (N₂O₄), S-nitrosoglutathione (GSNO), nitrogen dioxide radical (NO_2) , nitrosonium cation (NO⁺) and nitroxyl anion (NO⁻) (Wang *et al.*, 2013). Nitrosative stress, another stress process caused by environmental factors, evolves as the consequence of RNS accumulation in plant cells (Corpas et al., 2007, 2011). However, the two families of reactive molecules (ROS and RNS) are involved in overlapping signalling processes; indeed, the existence of nitrooxidative stress has been reported to occur under certain circumstances (Corpas and Barroso, 2013). An excellent example of ROS-RNS cross-talk is the reaction between O_2^{-} and NO yielding ONOO⁻, which is responsible for the protein tyrosine nitration which is becoming a useful biomarker of nitrosative stress in plants (Corpas et al., 2007, 2013). Protein tyrosine nitration is a post-translational modification resulting in the addition of a nitro group $(-NO_2)$ to one of the two equivalent ortho carbons in the aromatic ring of tyrosine residues (Gow et al., 2004). It causes steric and electronic perturbations, which modify the capability of tyrosine to function in electron transfer reactions or to maintain the proper protein conformation (van der Vliet et al., 1999). Tyrosine nitration can affect the function of a protein in numerous ways: in addition to no effect on functions or a gain function, the most common result of tyrosine nitration is the inhibition of the protein's function (Greenacre and Ischiropoulos, 2001; Radi, 2004). Furthermore, tyrosine nitration has the ability to influence several signal transduction pathways through the prevention of tyrosine phosphorylation (Galetskiy et al., 2011).

In most plants, the vacuoles of the root cells serve as the most important site of Zn storage, thus removing the metal from the root–shoot–leaf transport system and playing a crucial role in basal Zn tolerance (Arrivault *et al.*, 2006). Further protection mechanisms, such as alterations of the cell wall, e.g. callose deposition, facilitate the survival of the plants by limiting the uptake and translocation of heavy metals and by preventing the leakage of assimilates and other nutrients (Sjölund, 1997; Chen and Kim, 2009). During callose deposition, the properties of the cell wall are modified by adding extra layers of carbohydrates synthesized by callose synthase, a transmembrane protein in the outer plasma membrane (Kartusch, 2003).

Since heavy metals such as Zn result in a massive reduction in crop yield worldwide, the goal of this study was to investigate the morphological and physiological responses of two important crop plants, Indian mustard (*Brassica juncea*) and oilseed rape (*Brassica napus*), to Zn excess. Furthermore, our aim was to determine the potential involvement of ROS and RNS in the Zn sensitivity of *Brassica* species.

MATERIALS AND METHODS

Plant material and growing conditions

Brassica juncea (L.) Czern. and *Brassica napus* L. seeds were surface-sterilized with 5 % (v/v) sodium hypochlorite and then placed onto perlite-filled Eppendorf tubes floating on

full-strength Hoagland solution. The nutrient solution contained 5 mM Ca(NO₃)₂, 5 mM KNO₃, 2 mM MgSO₄, 1 mM KH₂PO₄, 0·01 mM Fe-EDTA, 10 μ M H₃BO₃, 1 μ M MnSO₄, 5 μ M ZnSO₄, 0·5 μ M CuSO₄, 0·1 μ M (NH₄)₆Mo₇O₂₄ and 10 μ M AlCl₃. Seedlings were pre-cultivated for 9 d – until the appearance of the first leaves – and then the nutrient solution was changed and supplemented with 0 (control), 50, 150 and 300 μ M ZnSO₄ for 7 d. Control plants were grown in full-strength Hoagland solution containing 5 μ M ZnSO₄. The plants were kept in a greenhouse at a photon flux density of 150 μ mol m⁻² s⁻¹ (12/12 h light/dark cycle) at a relative humidity of 55–60 % and 25 ± 2 °C for 7 d.

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

Element content analysis

The concentrations of microelements were measured by using inductively coupled plasma mass spectrometry (ICP-MS; Thermo Scientific XSeries II, Asheville, NC, USA) according to Lehotai *et al.* (2012). Root and shoot material of control, 50, 150 and 300 μ M Zn-treated *B. juncea* and *B. napus* were harvested separately and rinsed with distilled water to remove the potentially attached Zn from their surface. After 72 h of drying at 70 °C, 65 % (w/v) nitric acid and 30 % (w/v) H₂O₂ (both from Reanal, Budapest, Hungary) were added to the samples, which were subjected to 200 °C and 1600 W for 15 min. Values of Zn and other microelement concentrations are given in μ g g⁻¹ dry weight (d. wt).

Morphological measurements

Fresh weights (g) of the root material were measured on the seventh day of the treatment using a balance. The length of the primary root (PR; cm) and the first six lateral roots (LRs) from the root collar (cm) were also determined manually. Also the visible LRs were counted and their number is expressed as pieces per root. The root fresh weight and the PR length are expressed as a percentage of control.

Microscopic determination of Zn distribution, callose deposition, lipid peroxidation and loss of viability in the root tissues

For visualization of Zn, root tips were equilibrated in phosphate-buffered saline (PBS; 137 mM NaCl, 2.68 mM KCl, $8.1 \text{ mM} \text{ Na}_2\text{HPO}_4$, $1.47 \text{ mM} \text{ KH}_2\text{PO}_4$, pH 7.4), and further incubated with 25 μ M Zinquin [ethyl (2-methyl-8-*p*-toluenesulphonamido-6-quinolyloxy)acetate] in PBS for 1h at room temperature in darkness according to Sarret *et al.* (2006). Callose deposition in the root tissues was determined by image analysis using aniline blue according to Cao *et al.* (2011) with slight modifications. Root samples were incubated in aniline blue solution (0.1 %, w/v in 1 M glycine) for 5 min, and then washed once with distilled water. Products of lipid peroxidation [such as malondialdehyde (MDA)] were visualized using Schiff's reagent, according to Arasimowicz-Jelonek *et al.* (2009). Root tips were incubated in the dye solution for 20 min and then the reagent was replaced by 0.5 % (w/v) K₂S₂O₅ (prepared in 0.05 M HCl) for a further 20 min. For the determination of cell viability in the root tips, fluorescein diacetate (FDA) staining was used according to Lehotai *et al.* (2011). Root segments were incubated in 10 µM dye solution prepared in 10 mM MES (4-morpholine ethanesulphonic acid) buffer (pH 6.15) containing 50 mM KCl, then they were washed four times with MES/KCl.

Detection of ROS and RNS

Dihydroethidium (DHE) was used for visualization of superoxide anion contents in the root tips, which were incubated for 30 min in darkness at 37 °C in 10 µM dye solution and were washed twice with 10 mM Tris-HCl, pH 7.4 (Kolbert et al., 2012). For H_2O_2 detection, root segments were incubated in 50 µM AmplifluTM (10-acetyl-3,7-dihydroxyphenoxazine, ADHP or Amplex Red) solution and washed with 50 mM sodium phosphate buffer, pH 7.5, according to Lehotai et al. (2012). The NO levels in *Brassica* root tips were determined by 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM diacetate) (Kolbert et al., 2012). Root segments were incubated for 30 min in darkness at room temperature in 10 µM dye solution, and were washed twice with 10 mM Tris-HCl buffer, pH 7.4. Although DAF-FM diacetate allows only semiquantitative analysis, it is a reliable fluorophore for in situ detection of NO in plant tissues, since it does not react with H₂O₂ or peroxynitrite, but it responds to NO donors and/or scavengers (Kolbert et al., 2012). For the in situ and in vivo detection of peroxynitrite (ONOO⁻), 3'-(*p*-aminophenyl) fluorescein (APF) was applied (Chaki et al., 2009). The ONOOsensitivity of APF was proved in vitro, and it was also shown that the dye does not react with NO or H₂O₂ (Kolbert et al., 2012). Root samples were incubated in darkness at room temperature in 10 µM dye solution for 1 h and were washed twice with 10 mM Tris-HCl buffer, pH 7.4.

The roots of *Brassica* plants labelled with different fluorophores were investigated under a Zeiss Axiovert 200 M inverted microscope (Carl Zeiss, Jena, Germany) equipped with filter set 9 (excitation 450–490 nm, emission 515– ∞ nm) for DHE, filter set 10 (excitation 450–490, emission 515–565 nm) for APF, DAF-FM and FDA, filter set 20HE (excitation: 546/12 nm, emission 607/80 nm) for Amplex Red or filter set 49 (excitation: 365 nm, emission 445/50 nm) for aniline blue and Zinquin. Fluorescence intensities (pixel intensity) in the meristematic zone of the primary roots were measured on digital images using Axiovision Rel. 4.8 software within circles of 100 µm radius.

Visualization of intracellular zinc compartmentalization by confocal laser scanning microscopy (CLSM)

Root samples were washed three times alternately in deionized water and in 10 mM ethylenediaminetetraacetic acid (EDTA) before being incubated in 20 μ M Zinpyr-1 solution (in PBS) at room temperature in darkness for 3 h (Sinclair *et al.*, 2007). Samples were rinsed in deionized water, and immersed in 10 μ M propidium iodide (PI) to label cell walls (Tsukagoshi *et al.*, 2010). Samples were mounted in PBS and images were taken on a confocal laser scanning microscope (Olympus LSM 700, Olympus, Tokyo, Japan) using excitation at 488 nm with a 100 mW Ar ion laser and $a \times 20$ Plan Apo water immersion lens with fluorescein isothiocyanate (FITC) and PI filters. Images were processed with Olympus Fluoview FV100 software and were analysed using Fiji software (http://fiji.sc/Fiji; Schindelin *et al.*, 2012).

Measurement of the enzymatic antioxidant activity

Superoxide dismutase (EC 1.15.1.1) activity was determined by measuring the ability of the enzyme to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) in the presence of riboflavin in light (Dhindsa *et al.*, 1981). For the enzyme extract, 250 mg of plant material was ground with 10 mg of polyvinyl polypyrrolidone (PVPP) and 1 mL of 50 mm phosphate buffer (pH 7·0, with 1 mm EDTA added). The enzyme activity is expressed in U g⁻¹ fresh weight (f. wt); 1 U of SOD corresponds to the amount of enzyme causing a 50 % inhibition of NBT reduction in light.

The activity of APX (EC 1.11.1.11) was measured by monitoring the decrease of ascorbate content at 265 nm ($\epsilon = 14 \text{ mm}^{-1} \text{ cm}^{-1}$) according to a modification of the method of Nakano and Asada (1981). For the enzyme extract, 250 mg of plant material was ground with 1.5 mL of extraction buffer containing 1 mM EDTA, 50 mm NaCl and 900 μ M ascorbate. Data are expressed as activity (Ug⁻¹ f. wt).

SOD activity on native-PAGE, isoform staining

Isoforms of SOD were detected in gels by a modification of the method of Beauchamp and Fridovich (1971). SOD isozymes were separated by non-denaturating PAGE on 10 % acrylamide gels, followed by sequential incubation in 2·45 mM NBT for 20 min and in 28 μ M riboflavin and 28 mM tetramethyl ethylene diamine (TEMED) for 15 min in darkness. Colourless SOD bands on a dark blue background were observed after light exposure. SOD isoforms were identified by incubating gels in 50 mM potassium phosphate buffer (pH 7·0) supplemented with 3 mM KCN (inhibits Cu/Zn SOD) or 5 mM H₂O₂ (inhibits both Cu/Zn- and Fe-SOD) for 30 min before staining with NBT. Mn-SODs are resistant to both inhibitors.

Immunoprecipitation, SDS-PAGE and western blotting

Crude extracts from plant material were immunoprecipitated by using a Thermo Scientific Pierce Crosslink Magnetic IP/Co-IP Kit (Hudson, NH, USA). The beads were cross-linked with antibody against 3-nitrotyrosine. After purification, immunoprecipitated samples were subjected to SDS–PAGE on 12 % acrylamide gels. For western blot analysis, proteins were transferred to polyvinylidene fluoride (PVDF) membranes using the wet blotting procedure. After transfer, membranes were used for cross-reactivity assays with rabbit polyclonal antibody against 3-nitrotyrosine diluted 1:2000 (Corpas *et al.*, 2008). Immunodetection was performed by using affinity-isolated goat anti-rabbit IgG–alkaline phosphatase secondary antibody at a dilution of 1:10 000, and bands were visualized by using the Page 4 of 13

NBT/BCIP reaction. As a positive control, nitrated bovine serum albumin was used.

Statistical analysis

All experiments were carried out at least twice. In each treatment, at least 10–20 samples were measured. The results are expressed as the mean \pm s.e. Multiple comparison analyses were performed with SigmaStat 12 software using analysis of variance (ANOVA; P < 0.05) and Duncan's test. In some cases, Microsoft Excel 2010 and Student's *t*-test were used (* $P \le 0.05$, ** $P \le 0.01$, *** $P \le 0.001$).

RESULTS AND DISCUSSION

Zinc uptake, accumulation and microelement homeostasis in Brassica species

Increasing Zn concentrations in the nutrient solutions promoted significant increases of Zn content of the root system in both species (Fig. 1A). The two *Brassica* species showed no differences in their Zn uptake capacity, although in the case of the highest Zn treatment *B. juncea* accumulated slightly (approx. 14 %) more metal in its root system. With regard to the shoots, the treatments resulted in a concentration-dependent response of Zn content; however the values were lower by one order of



FIG. 1. Zinc concentration ($\mu g g^{-1} d$. wt) in roots (A) and shoots (B) of control and zinc-exposed *B. juncea* and *B. napus* plants. Significant differences with respect to control plants (5 μm ZnSO₄) and according to Student's *t*-test (n = 10, *** $P \le 0.001$) are indicated.

magnitude than in the root. Moreover, the shoot system of B. napus contained higher (approx. 48, appprox. 33 and approx. 14 %, respectively) Zn levels compared with that of B. juncea (Fig. 1B). The results suggest an efficient root to shoot Zn translocation in both species; however, B. napus showed a better transport capacity to the aerial parts in the case of all Zn treatments. Zinc is predominantly complexed with citric and malic acid in the xylem sap. Moreover, small amounts of soluble Zn-phosphate were also found in the sap in the case of excess Zn (White et al., 1981). In our experimental system, the Brassica species at their early developmental stage (16-day-old plants) proved to be Zn accumulators, since the amount of transported Zn is >0.1 % of the shoot dry weight. Similar Zn accumulation tendencies were found in the roots and shoots of 12-day-old Brassica ssp., which were considered to be moderate Zn accumulators with some potential for phytoremediation (Ebbs and Kochian, 1997).

In addition to Zn, the concentrations of other microelements [copper (Cu), manganese (Mn) and iron (Fe)] were also determined in the roots of *Brassica* species using the ICP-MS technique (Table 1). The applied Zn treatments resulted in elevated Cu contents in the root system of both Brassica species compared with the control. The lack of Zn-Cu antagonism can be explained by the discrimination between the divalent ions (Irving–Williams order). Zn and Cu use the same transporters, which can be upregulated by Zn excess, although they prefer Cu more than Zn (Fraústo da Silva and Williams, 2001). This can trigger the increase in Cu content in the two Zn-exposed Brassica species. Similarly to Cu, Fe contents of the root system showed a slight but significant increase in both species as was the case for Zn. In contrast, all the applied Zn concentrations led to a strong decrease in Mn content in the roots of both species. In Arabidopsis thaliana and Thlaspi caerulescens roots, Zn caused similar changes in Fe and Mn contents (van de Mortel et al., 2006). Moreover, in the roots of Zn-exposed Lolium perenne the Mn contents were also significantly reduced (Monnet et al., 2001). The synergistic effect we found between Fe and Zn suggests that both species may increase their Fe uptake in order to avoid Fe deficiency in leaves. In Arabidopsis roots, Zn excess notably induced the expression of the ferric-chelate reductase gene (FRO2), which contributed to the intensification of Fe uptake (van de Mortel et al., 2006). In the case of Mn, an antagonistic relationship with Zn seems to be the case in the roots of both species.

TABLE 1. Copper (Cu), manganese (Mn) and iron (Fe) concentrations ($\mu g g^{-1} d$. wt) in roots of control and zinc-treated Brassica plants

	Control	50 µм Zn	150 µм Zn	300 µм Zn
Brass	sica juncea			
Cu	24.50 ± 0.38	173.00 ± 0.52	198.90 ± 0.49	174.80 ± 0.73
Mn	250.00 ± 1.16	24.97 ± 0.05	25.53 ± 0.07	26.02 ± 0.13
Fe	519.10 ± 4.75	864.50 ± 3.65	869.90 ± 2.71	927.90 ± 2.22
Brass	sica napus			
Cu	27.68 ± 0.08	150.60 ± 0.50	178.60 ± 0.85	163.70 ± 0.68
Mn	145.10 ± 0.53	26.40 ± 0.09	22.02 ± 0.10	26.44 ± 0.17
Fe	1051.0 ± 4.5	1496.0 ± 6.7	1540.0 ± 11.3	1239.0 ± 7.8

Values are significantly different from the controls in both species and in case of all elements at $P \le 0.001$.

Tissue-specific and sub-cellular localization of zinc in the root of **Brassica** *species*

The increase in the fluorescent signal was not concentration dependent in either *B. juncea* or *B. napus* (Fig. 2B).

The tissue localization pattern of Zn in the root tips was visualized by the Zinquin fluorophore. Homogenous, low-level Zndependent fluorescence was detected in the root tips of control plants. With the increasing external Zn concentration, the accumulation of the fluorescent signal was most evident in the meristematic and transition zones (Fig. 2), probably because of the greater permeability of the thin walls of meristem cells to Zn ions. In the cells of the elongation zone, a lower fluorescence intensity was observed, while in the differentiation zone the Znassociated fluorescence intensified as a result of external treatments and the root hairs also showed Zn content (see Fig. 2A). Similarly, in the root tips of Solanum nigrum, Zn exposure (400 µM ZnCl₂) caused the intensification of Zinquin fluorescence, but this fluorescent signal showed a homogenous distribution within the tip (Xu et al., 2010). Although there was practically no difference between the Zn contents of the whole root system of the species (see Fig. 1A), the Zinquin fluorescence of the meristematic zones proved to be higher in Zn-treated B. juncea (Fig 2B), which suggests the higher accumulation of Zn in the root tips compared with B. napus.

The sub-cellular Zn distribution within the root cells of Zntreated Brassica was investigated by confocal microscopy in order to reveal the role of the cell wall in Zn binding. The Zn-dependent green fluorescence was most intense in the walls of the epidermal cell layer of the root. Zinc also localized in the cvtoplasm and/or in vacuoles and around the surface of the nuclei of these cells, which also showed PI-dependent fluorescence, suggesting that these cells are not viable. The localization of Zn in the root cell nuclei of Zn-exposed plants was also shown by Rathore et al. (1972). In the inner cell layers of the root, mainly the apoplast showed Zn content, and most of the cells were alive (Fig. 3). Similarly to the results of Küpper et al. (2000), root epidermal cells accumulate Zn mostly in their walls. The cell wall metabolic inactivity provides advantage for metal precipitation and exclusion from the cytoplasm (Krzesłowska, 2011), which can ensure the survival in the case of metal excess (Rout and Das, 2003). Some early studies suggested that Zn is associated with the carbohydrate components of the cell wall such as hemicelluloses and pectins (Diez-Altares and Bornemisza, 1967; Turner and Marshall, 1972). Recent studies revealed that low-methylesterified pectins are



Fig. 2. (A) Tissue specificity of zinc localization in control and zinc-treated *Brassica juncea* and *Brassica napus* root tips. Zinc localization was visualized with the aid of the Zinquin fluorophore as described in the Materials and Methods. Scale bar = $200 \,\mu\text{m}$. (B) Pixel intensities of Zinquin fluorescence measured in the PR meristem of control and zinc-treated *Brassica* species. Different letters indicate significant differences according to Duncan's test ($n = 10, P \le 0.05$).



FIG. 3. Confocal microscopic images of the root tip of 300 μ M zinc-treated *B. juncea*. The root samples were co-stained with Zinpyr-1 and PI. (A) Lower magnification merged image of the whole root tip (A) and enlarged image showing root epidermal cells (B). Green (Zinpyr-1) fluorescence corresponds to zinc (C) and red (PI) fluorescence shows cell walls and nuclei (D). Scale bars = 100 μ m.

the most important metal-binding components of the cell wall (Krzesłowska, 2011). The epidermal cells suffer from cell death presumably because of the presence of Zn also in their cytoplasm, while the inner cells (cortex) contain less Zn mainly in their walls, and they remain viable.

Zinc-triggered changes in root architecture

With the increasing Zn concentrations, leaf area, and the fresh and dry weight of the shoot significantly decreased (data not shown) and chlorosis was also visible (Fig. 4A). However, necrotic lesions on the leaf blades were not observed during the experimental period. The root system is of great importance during the life of heavy metal-exposed plants, since it can contribute to tolerance, e.g. by controlling metal uptake or storage of excess metal. This supported the need for a detailed investigation of root development, which revealed differences between the Brassica species. The root tip morphology was modified by Zn excess, since the meristematic and transition zones were narrower, while the diameter of the upper regions was visibly larger than in the control root (see Fig. 2A). This Zn-induced morphological alteration was observed in both species, but it was more evident in the case of B. napus. Moreover, root hair formation was remarkably induced by Zn excess, particularly in *B. napus* roots (see Fig. 2A). Interestingly, the PR elongation of *B. juncea* was not notably affected by Zn, while in the case of *B. napus* it was significantly inhibited at all applied Zn concentrations (Fig. 4B). Mild stress (50 µM ZnSO₄) resulted in a notable elevation of LR number in both species. Similarly, 150 µM ZnSO₄ increased the number of LRs, but the effect was much less in this case. Moreover, the highest applied Zn concentration did not affect the LR development of either

species (Fig. 4C). The length of the LRs was remarkably diminished by Zn exposure in both *Brassica* species: however, the rate of inhibition proved to be lower in 50 µM Zn-treated B. juncea than in B. napus (Fig. 4D). Based on these data, the cell elongation and division processes in the PRs and LRs are more sensitive to Zn excess than the anticlinal divisions of the pericycle cells during LR initiation. Indeed, the PR tips of Zn-treated sugarcane showed a significantly reduced mitotic index and a wide spectrum of cytotoxic effects (Jain et al., 2010). The fresh weight of the root system showed Zn-induced reduction only in B. napus, while in B. juncea Zn stress was not able to alter the fresh root biomass (Fig. 4E). The results show that Zn excess modifies the root system architecture depending on its concentration, and the effect was different in the two species. Mild Zn exposure (50 µM ZnSO₄) triggered the development of the stressinduced morphogenetic response (SIMR) phenotype (Potters et al., 2009) only in B. napus, since it resulted in a shorter PR and higher number of (shorter) LRs. Similar stress-induced root development was observed, e.g. in selenium- (Se) or Cu-exposed Arabidopsis or chromium- (Cr) treated wheat plants (Hasnain and Sabri, 1997; Pető et al., 2011; Lehotai et al., 2012). In contrast, in *B. juncea*, LR formation was induced (more significantly than in *B. napus*) and the PR elongation was not affected by Zn stress, which led to the development of an extended root system compared with the control plants. It can be assumed that these developmental changes are part of the acclimation process because they can ensure better nutrient and water uptake and thus survival of the *B. juncea* plant.

Zinc stress provokes changes in the cell wall structure

Cell wall alterations, such as lignification or callose deposition, can help the plant cells to tolerate excess heavy metal by serving as a physical barrier, thus preventing the heavy metals from entering the cytoplasm. In addition to their role in heavy metal tolerance, these cell wall modifications can also be partly responsible for decreased growth.

Under Cu stress, H₂O₂-dependent lignin formation in the LRs of both Brassica species was found (Feigl et al., 2013), but Zn-induced lignification was not detectable in the root system (data not shown). On the other hand, the results show that excess Zn caused significant callose deposition in the roots of both species and this callose content increment was more pronounced in B. napus (Fig. 5). Similarly, callose accumulation was observed in Zn-treated bean plants (Peterson and Rauser, 1979). The deposited callose could inhibit root growth by decreasing cell wall loosening, thus preventing the passage of signal molecules or inhibiting the symplastic supply of carbon required for root growth (Jones et al., 2006; Piršelová et al., 2012). In comparison, there was no significant callose deposition induced by Cu stress either in *B. juncea* or in *B. napus* (Feigl et al., 2013), so thus it can be stated that this cell wall modification is a heavy metal-dependent process, but it is independent of the plant species.

The Brassica species show different sensitivity to zinc stress

We characterized the degree of Zn sensitivity by detecting the viability of the root meristem (FDA labelling) and



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FIG. 4. The effect of excess zinc on the growth of *B. juncea* and *B. napus* plants. (A) Representative photographs of 16-day-old untreated and zinc-exposed *B. juncea* and *B. napus* plants. Scale bar = 1 cm. Primary root length as a percentage of control (B), number (C) and length (D) of lateral roots, and root fresh weight as a percentage of control (E) of *Brassica* plants treated with 0, 50, 150 or 300 μ m zinc. The lack of significance (n.s.) or significant differences according to Student's *t*-test (n = 20, ** $P \le 0.01$, *** $P \le 0.001$) are indicated.

calculating the tolerance index (%) based on PR elongation. The root meristem cells of *B. juncea* remained fully viable even in case of 300 μ M ZnSO₄ treatment, while the root meristem of *B. napus* underwent significant loss of viability as a result of Zn exposure (Fig. 6). Based on the results, the viability status of the PR meristem cells is in accordance with the elongation capability of the root (see Fig. 4B). The tolerance indexes of Zn-treated *B. juncea* showed no decrease at higher concentrations of external Zn (control, 100 %; 50 μ M ZnSO₄, 97 %; 150 μ M ZnSO₄, 119 %; and 300 μ M ZnSO₄, 107 %); however, they decreased significantly in the case of Zn-exposed *B. napus* (control, 100 %; 50 μ M ZnSO₄, 61 %; 150 μ M ZnSO₄, 50 %; and 300 μ M ZnSO₄, 52 %). The results show that *B. juncea* possesses remarkable Zn tolerance compared with

B. napus, which supports the species specificity of zinc sensitivity.

Altered metabolism of ROS and RNS in Zn-exposed Brassica species

The effect of Zn excess on the levels of ROS, RNS and antioxidants was determined in this work. In the roots of *B. juncea*, the level of superoxide anion significantly decreased as a consequence of Zn excess (Fig. 7A), which can be explained by the enhancement of SOD activity (Fig. 7B). In contrast, superoxide levels of 50 and 150 µm Zn-treated *B. napus* roots showed a significant increment, which was accompanied by increased SOD



Fig. 5. Intensity of aniline blue-dependent fluorescence (as a percentage of control) reflecting callose levels in the root tips of control and zinc-treated *Brassica* plants. Significant differences according to Student's *t*-test (n = 10, $*P \le 0.05$, $**P \le 0.01$, $**P \le 0.001$) are indicated.



FIG. 6. Viability (percentage of the fluorescein pixel intensity in control) of the root meristem cells of control and zinc-exposed *Brassica* plants. The lack of significance (n.s.) or significant differences according to Student's *t*-test (n = 10, $*P \le 0.05$, $**P \le 0.01$, $***P \le 0.001$) are indicated.

activity. These findings suggest that the elevated SOD activity was not able to compensate the formation of superoxide anion in the case of 50 and 150 µM Zn, although it could reduce the superoxide content during severe Zn stress (300 µM). We separated the different SOD isoforms by native-PAGE, and five activity bands were identified in the case of both species (Fig. 8). The pattern obtained is in agreement with the result published by Cohu and Pilon (2007) in the case of B. juncea; however, we found a different configuration of SOD isoforms in B. napus compared with what was found by Abedi and Paniyat (2010). The experiments with specific inhibitors showed that the uppermost band represented an Mn-SOD isoform, whose activity decreased due to the increasing Zn concentrations in both species, but particularly in *B. napus*. The decrease of Mn-SOD activity can be explained by the reduced availability of Mn as previously shown in Table 1. The Fe-SOD isoform was hardly visible in the case of B. juncea and only present in the control sample of B. napus. The last three bands showed Cu/Zn-SODs, whose strengths were correlated with the overall SOD activity (see Fig. 6B). Similarly to our results, the decrease in the activity of all three isoenzymes has been shown previously, e.g. in cadmium-exposed pea plants (Sandalio *et al.*, 2001).

The level of H_2O_2 remained low in Zn-treated *B. juncea* (Fig. 7C), and the pattern of APX activity (Fig. 7D) could partly explain the H_2O_2 profile. In contrast, in *B. napus* the highest applied Zn concentration resulted in an extreme H_2O_2 accumulation, but APX did not vary compared with control plants (Fig, 7C and D, respectively). Zn-triggered ROS formation and modification of antioxidant capacity have been reported previously, e.g. in sugarcane, bean, maize and pea (Chaoui *et al.*, 1997; Lozano-Rodríguez *et al.*, 1997; Jain *et al.*, 2010).

In the root tips of both examined species, NO formation was detectable in a concentration-dependent manner; however, this elevation was statistically significant only in the roots of B. juncea (Fig. 7E). There are several possible mechanisms of NO formation in this system. Xu et al. (2010) reported that Zninduced Fe deficiency can be partially responsible for NO production in Solanum nigrum root tips, although in our experiments, Zn-induced Fe deficiency was not observed (see Table 1). The major enzymatic source of NO in the roots is nitrate reductase, but this activity was not influenced by Zn excess in Brassica roots (Bartha et al., 2005). Furthermore, the transition metal-triggered decomposition of NO pools such as GSNO (Smith and Dasgupta, 2000) may result in NO liberation in Zn-exposed Brassica roots, but this possibility remains to be examined. Nitric oxide may react with superoxide anion yielding peroxynitrite (ONOO⁻), a powerful oxidative and nitrosative agent (Arasimowicz-Jelonek and Floryszak-Wieczorek, 2011). The significant Zn-induced enhancement of peroxynitrite content in both species (Fig. 7F) may explain the moderate NO accumulation, since part of the NO formed was possibly transformed into peroxynitrite. This hypothesis can be supported by the decreasing superoxide levels in B. juncea (Fig 7B); while in *B. napus* superoxide levels remained high (Fig. 7B) and less peroxynitrite is perhaps being produced through this pathway (Fig. 7F). The SOD system is possibly playing an important role in the regulation of peroxynitrite formation, by modulating the levels of superoxide radicals in this reaction. The representative fluorescent microscopic images of the root tips stained with different fluorophores can be seen in Fig. 7G.

The significant and Zn concentration-dependent peroxynitrite formation in both species predicted protein tyrosine nitration and, therefore, this event was studied by western blot analysis using an antibody against nitrotyrosine (Fig. 9). The presence of seven nitrotyrosine-immunopositive protein bands in the untreated samples suggests that a part of the protein pool is nitrated even under control circumstances. Similarly, a basal nitration state of proteins was reported in different plant species such as sunflower, Citrus and pea (see Chaki et al., 2009; Begara-Morales et al., 2013; Corpas et al., 2013). We observed strengthening of the same seven protein bands due to the effect of $300 \,\mu\text{M}$ ZnSO₄, which suggests the intensification of protein nitration induced by Zn excess. The enhancement of nitration levels was pronounced in both species, which implies that the proteome of both species is sensitive to nitrosative modification. Similarly, intensified tyrosine nitration was observed in salt-stressed olive leaves as well as in leaves of cold-treated pea



FIG. 7. Effect of Zn excess on the metabolism of ROS and RNS. The levels of superoxide radicals (pixel intensity of DHE fluorescence; A), total SOD activity (B), H₂O₂ (pixel intensity of resorufin fluorescence; C), APX activity (D), NO (pixel intensity of DAF-FM fluorescence; E) and ONOO⁻ (pixel intensity of APF fluorescence; F) in control and zinc-treated *Brassica* roots (in meristematic zone) are shown. (G) Representative microscopic images of *Brassica* root tips stained with different fluorophores (from left: DHE for superoxide in *B. napus*, AmplifluTM for H₂O₂ in *B. napus*, DAF-FM for NO in *B. napus*, APF for ONOO⁻ in *B. napus*). The fluorescent staining procedures were carried out in the root tips, while enzymatic activities were measured in the whole root system as described in the Materials and Methods. Different letters indicate significant differences according to Duncan's test ($n = 10, P \le 0.05$).



Fig. 8. Analysis of SOD isoforms. SOD isoforms were separated by native-PAGE and stained by a photochemical method using 30 µg of protein per lane.



Fig. 9. Representative immunoblots showing protein tyrosine nitration in roots of *B. juncea* and *B. napus* plants under control conditions and subjected to 300 µM Zn. Root samples were separated by SDS–PAGE and analysed by western blotting with anti-nitrotyrosine antibody (1:2000). Commercial nitrated bovine serum albumin (NO₂-BSA) was used as a positive control.

or in water-stressed *Lotus japonicus* (Valderrama *et al.*, 2006; Corpas *et al.*, 2008; Signorelli *et al.*, 2013); however, to our knowledge, this is the first study which demonstrates heavy metal-induced protein nitration.

Peroxynitrite – through the formation of peroxynitrous acid (ONOOH) – can lead to lipid peroxidation, whose product – MDA – can be detected *in situ* histochemically (Arasimowicz-Jelonek *et al.*, 2009). During the microscopic investigation using the Schiff's staining procedure, the root tips of *B. napus* showed slight but visible pink coloration reflecting the Zn-induced increase in the MDA content (Fig. 10). In contrast, Zn-treated *B. juncea* root tips remained unstained. These findings suggest that the root tip cells of *B. napus* suffered oxidative membrane damage, while in the root tips of *B. juncea* there was no detectable lipid peroxidation.



Fig. 10. Microscopic images of Brassica root tips stained with Schiff's reagent. The pink coloration indicates lipid peroxidation. Scale bar = 200 µm.

Conclusions

Taken together, these results clearly show that the morphological and physiological responses of Brassica species to Zn stress are different. In the roots of B. juncea, which possesses better Zn resistance, only a slight ROS formation, activation of antioxidant enzymes (SOD, APX) and no remarkable lipid peroxidation were observed, which reflect the lack of a Zn-induced serious oxidative stress. However, the significant production of RNS (NO and ONOO⁻) and the occurrence of protein nitration reveal a Zn-triggered secondary, nitrosative stress in B. juncea. In contrast, as a result of Zn exposure, nitro-oxidative stress occurred in the more sensitive B. napus as a consequence of ROS and RNS accumulation, lipid peroxidation and protein tyrosine nitration. Our data reveal the existence of a relationship between ROS and RNS metabolism under Zn stress and the contribution of nitro-oxidative stress to Zn sensitivity. The results also suggest that sensitivity to Zn is determined by the level of oxidative rather than by the nitrosative processes in Brassica species.

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