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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.

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 ($Yield = (Fm' - Ft)/Fm'$), electron transport rate ($ETR = Yield \times PAR \times 0.5 \times 0.84$), 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.

The viability of the root meristem cells was determined using 10 μM fluorescein diacetate (FDA) solution (in 10/50 mM MES/KCl buffer, pH 6.15) at room temperature (25 ± 2 °C) in the dark (Lehotai et al., 2012).

The level of superoxide anion in the root tip was estimated using 10 μM dihydroethidium (DHE) (prepared with 10 mM Tris/HCl, pH 7.4) in the dark at 37 °C. (Kolbert et al., 2012).

For hydrogen peroxide detection, root tips were incubated in 50 μM Ampliflu™ (10-acetyl-3,7-dihydroxyphenoxazine, ADHP or Amplex Red) solution at room temperature in the dark for 30 min according to Lehotai et al. (2012).

The fluorophore, 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA, 10 μM in 10 mM Tris/HCl buffer, pH 7.4) was applied for the visualization of NO levels in *Brassica* root tip segments (Kolbert et al., 2012).

For the in situ and in vivo detection of peroxynitrite ($ONOO^-$), 10 μM 3′-(*p*-aminophenyl) fluorescein (APF) was applied according to Chaki et al. (2009). Although these staining methods allow semi-quantitative determinations, they are reliable tools for in situ detection of ROS and RNS, since their specificity were proved in vivo and in vitro (Kolbert et al., 2012).

The roots of the plants labelled with different fluorophores were investigated under a Zeiss Axiovert 200M inverted microscope (Carl Zeiss, Jena, Germany) equipped with filter set 9 (exc.: 450–490 nm, em.: 515- ∞ nm) for DHE, filter set 10 (exc.: 450–490, em.: 515–565 nm) for APF, DAF-FM and FDA, filter set 20HE (exc.: 546/12, em.: 607/80) for Amplex Red. Digital photographs from the samples were taken by a digital camera (Axiocam HR, HQ CCD). The same camera settings were applied for each digital image. In all cases, 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 radii. At least 10–15 root tips were measured in each experiment.

2.8. Measurement of the enzymatic antioxidant activity and lipid peroxidation

The activity of superoxide dismutase (EC 1.15.1.1) was determined by measuring the ability of the enzyme to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) in the presence of riboflavin, in light (Dhindsa et al., 1981). For the enzyme extract, 250 mg fresh plant material was grinded with 10 mg polyvinyl polypyrrolidone (PVPP) and 1 ml 50 mM phosphate buffer (pH 7.0, with 1 mM EDTA added). The enzyme activity is expressed in Unit/g fresh weight; one unit (U) of SOD corresponds to the amount of enzyme causing a 50% inhibition of NBT reduction in light.

Ascorbate peroxidase (APX; EC 1.11.1.11) activity was measured by monitoring the decrease of ascorbate content at 265 nm ($\varepsilon = 14\ mM^{-1}\ cm^{-1}$) according to a modified method by Nakano and Asada (1981). For the enzyme extract, 250 mg fresh plant material was grinded with 1.5 ml extraction buffer containing 1 mM EDTA, 50 mM NaCl and 900 μM ascorbate. Data are expressed as activity (Unit/g fresh weight).

The zinc-induced lipid peroxidation in the root and shoot tissues was quantified by the measurement of thiobarbituric acid reactive substances (TBARS) concentration (Heath and Packer, 1968). 100 mg of shoot and root tissues were freshly grounded in liquid nitrogen, suspended in 1 ml 0.1% tri-chloro acetic acid (TCA), and then centrifuged at 12.000 rpm for 20 min in the presence of butylated hydroxytoluene (BHT) (0.1 ml, 4%) to prevent further lipid peroxidation. 250 μl of the supernatant was removed and incubated at 100 °C for 30 min with 1 ml of 0.5% 2-thiobarbituric acid (TBA) dissolved in 20% TCA. After cooling the samples on ice, they were refilled to the starting volume. The absorbance of the supernatant was determined at 532 nm, and corrected for unspecific turbidity after subtraction from the value obtained at 600 nm. The level of lipid peroxidation is expressed as nmol TBARS per gram fresh weight, using an extinction coefficient of $155\ mM^{-1}\ cm^{-1}$.

2.9. SOD activity on native PAGE, isoform staining

The isoforms and activity of SOD (Mn-SOD, Fe-SOD, Cu/Zn-SODs) were detected in gel according to the modified method of Beauchamp and Fridovich (1971). After the separation of SOD isozymes by non-denaturating PAGE on 10% acrylamide gels, they were incubated sequentially in 2.45 mM NBT for 20 min and in 28 μM riboflavin and 28 mM tetramethyl ethylene diamine (TEMED) for 15 min in the dark. After light exposure, the colourless SOD bands were observed on a dark blue background. The different isoforms were identified by incubating the gels in 50 mM potassium phosphate buffer (pH 7.0) supplemented with 3 mM KCN (inhibits Cu/Zn SOD) or 5 mM H_2O_2 (inhibits both Cu/Zn- and Fe-SOD) for 30 min before staining with NBT. Mn-SODs are resistant to both inhibitors.

2.10. Preparation of protein extract

Shoot and root tissues of *Brassica* species were grounded with double volume of extraction buffer [50 mM Tris–HCl buffer (pH 7.6–7.8) containing 0.1 mM EDTA (ethylenediaminetetraacetic acid), 0.1% Triton X-100 [polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether) and 10% glicerol]. After centrifugation at 12,000 rpm for 20 min at 4 °C, the supernatant was stored at −20 °C. Protein concentration was determined using the Bradford (1976) assay with bovine serum albumin as a standard.

2.11. SDS-PAGE and western blotting

10 μg of root and 25 μg of shoot protein extracts per lane were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% acrylamide gels. For western blot analysis, separated proteins were transferred to PVDF membranes using the wet blotting procedure (30 mA, 16 h). 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 in dilution of 1:10 000, and bands were visualised by using NBT/BCIP reaction. As a positive control nitrated bovine serum albumin was used.

2.12. Statistical analysis

All experiments were carried out at least two times. The results are expressed as mean ± SE. Multiple comparison analyses were performed with SigmaStat 12 software using analysis of variance (ANOVA, $P < 0.05$) and Duncan's test.

3. Results and discussion

3.1. Zinc accumulation and translocation capacity of Brassica species are similar

As the effect of increasing external zinc sulphate concentrations, the zinc content of the root system of both species dramatically increased (Fig. 1a). The roots of *B. napus* showed maximal accumulation already at 150 μM Zn, while in *B. juncea* roots, by 60% lower zinc concentration was measured at this treatment. This indicates that *B. napus* roots possess a more efficient zinc uptake system compared to *B. juncea*. Moreover, the enhancement of zinc

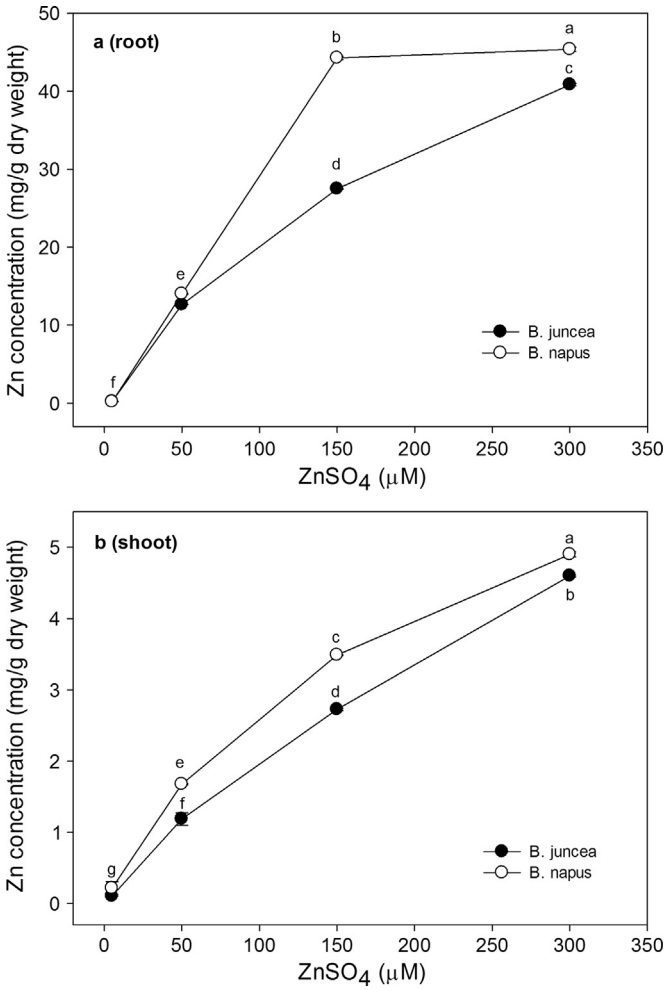


Fig. 1. Concentration of zinc (μg/g dry weight) in the root (a) and shoot (b) system of 0, 50, 150 or 300 μM $ZnSO_4$-treated *B. juncea* (•) and *B. napus* (○). Different letters indicate significant differences according to Duncan-test ($n=6$, $P \leq 0.05$).

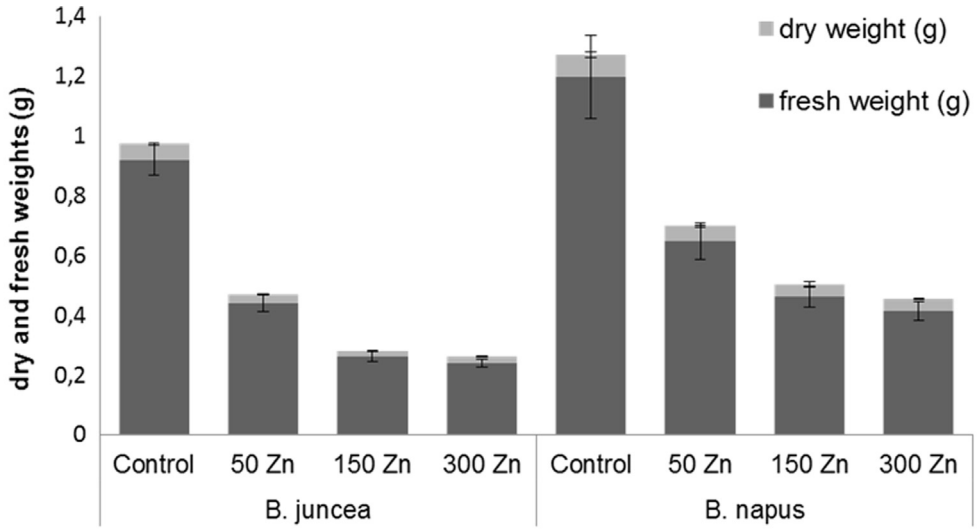
concentration in the root tissues of *B. juncea* proved to be directly proportional to the external zinc concentration of the nutrient solution ($R^2=0.999$). In the aerial plant parts, as the effect of external exposure the zinc concentration significantly enhanced (Fig. 1b) in both species, suggesting that root-to-shoot transport occurs. The most abundant transport forms of zinc are complexes with citric, malic and oxalic acid (Lu et al., 2013). According to the results of White et al. (1981), small amounts of soluble zinc-phosphate can also be found in the xylem sap of zinc stressed plants. However, it has to be noted that in the shoot tissues, an order of magnitude lower zinc contents were measured compared to the root. This suggests that the zinc translocation capability of the *Brassica* species is relatively poor, which can be a part of an exclusion defence strategy (Baker, 1987). With the restriction of its root-to-shoot translocation, plants try to protect the more sensitive shoot from zinc-induced damages. At the same time, both species accumulated more zinc than 0.1% of the shoot dry weight (0.45% of shoot DW in *B. juncea* and 0.49% of shoot DW in *B. napus*); therefore both species are considered to be zinc accumulators. In other works, similar Zn accumulation tendencies were observed in the *Brassica* species, and they were considered to be as moderate zinc accumulators (Kumar et al., 1995, Ebbs et al., 1997; Ebbs and Kochian, 1997).

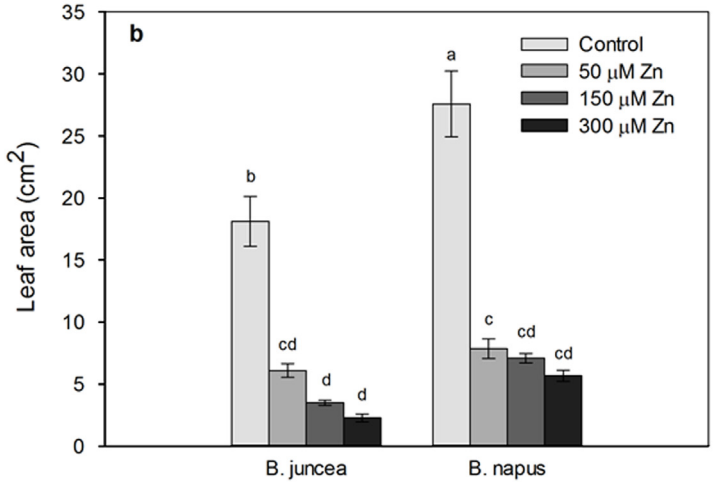
3.2. Excess zinc induces similar disturbances in the microelement homeostasis of Brassica species

Besides zinc, the concentrations of iron (Fe), manganese (Mn), boron (B), copper (Cu), molybdenum (Mo) and nickel (Ni) were also measured by ICP-MS, in order to evaluate the putative disruption in microelement homeostasis provoked by zinc exposure (Supplementary Fig 1). Surprisingly, excess zinc led to the increase of copper content in both organs of both species. This can be explained by that both ions use the same transporters, which can be up-regulated by excess Zn, although they prefer Cu (Fraústo da Silva and Williams, 2001) provoking the increase of Cu content in the Zn-exposed plants. The manganese concentration was found to be remarkably decreased in the organs of Zn-exposed *Brassica* species, which suggests an antagonistic relationship between the two ions. Similarly to our results, in the shoots of zinc-exposed *B. juncea* and *B. napus* cultivars and in the roots of *Lolium perenne* the Mn contents were significantly reduced (Ebbs and Kochian, 1997; Monnet et al., 2001). Decrease in manganese content may due to competition of zinc with manganese for transport sites in the plasmalemma. The concentrations of iron and boron were differentially influenced by zinc treatment in the organs. A notable zinc-induced loss of Fe content was observed in the shoot tissues of both species. In the case of *B. juncea*, the concentration of Fe ion remarkably increased within the root system, but it was not modified in the roots of *B. napus*. The synergistic effect between iron and zinc observed in *B. juncea* roots suggests that this species may intensify their iron uptake into the root in order to compensate iron diminution in leaves. In *Arabidopsis* roots, excess Zn 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). Although, the inhibitory effect of excess zinc on the root-to-shoot Fe translocation was also evidenced e.g. in soybean, Japanese mint or *Picea abies* (Ambler et al., 1970; Misra and Ramani, 1991; Godbold and Huttermann, 1985), which may provide a possible explanation for the altered Fe distribution between the organs of *B. juncea*. Excess zinc modified the concentration of B in the organs of both species as well, and within the shoot system, the enhancement of B content was worth mentioning. Similar synergism between boron and zinc was observed in mustard by Sinha et al. (2000).The increase of Mo contents was evident in the shoot of Zn-exposed *Brassica*, and it was not modified within the root system. Moreover, Zn exposure did not significantly altered Ni concentrations of the *Brassica* organs. The observed changes in microelement concentrations and distribution suggest that excess zinc is able to disrupt the homeostasis of micronutrients in the organs by interfering with their uptake, translocation and metabolism (Stoyanova and Doncheva, 2002). We observed similar changes in the *Brassica* species, which supports the species-independent rather general nature of the zinc-triggered micronutrient disturbances.

3.3. Growth and morphology of Brassica organs are differentially affected by excess zinc

During control circumstances, the shoot system of *B. napus* proved to be more extended than that of *B. juncea*, which is indicated by the significantly higher fresh, dry biomass and the larger leaf area of it (Fig. 2a, b and c, respectively). As the effect of 50 and 150 μM Zn, concentration-dependent decrease of shoot FW was observed in both species (Fig. 2a). The most serious Zn exposure (300 μM) did not reduce the biomass further compared to the 150 μM Zn treatment. Regarding the shoot DW (Fig. 2a), Zn at all concentrations reduced it significantly compared to the control. In case of both fresh and dry biomass, the species were differentially affected, since in *B. juncea,* Zn resulted in 78% reduction of shoot FW and in 60% of shoot DW, but *B. napus* showed only 64%





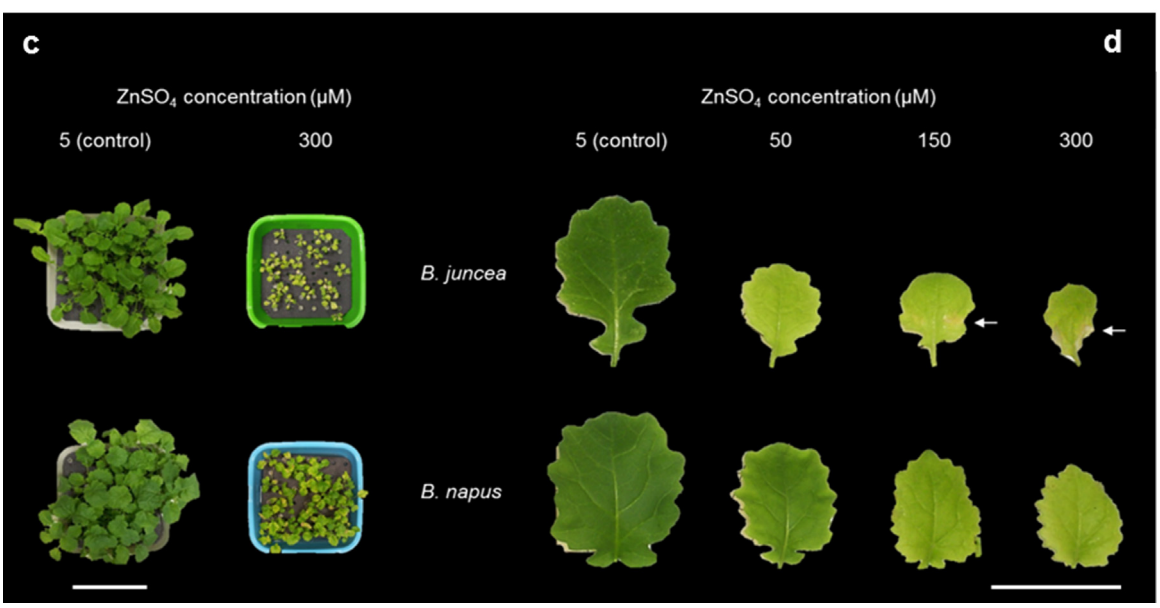


Fig. 2. (a) Dry and fresh weight (g) of the shoot system of *Brassica* species treated with 0, 50, 150 or 300 μM Zn. (b) Leaf area (cm^2) of control and Zn-exposed *Brassica* plants. Different letters indicate significant differences according to Duncan-test ($n=20$, $P \leq 0.05$). (c) Photographs taken from the shoot system of control and 300 μM Zn-treated *B. juncea* and *B. napus*. Bar = 30 cm. (d) Representative photographs of *Brassica* leaves demonstrating the effect of zinc concentrations on morphology and on the appearance of chlorosis and necrosis (marked by white arrows). Bar = 5 cm.

and 43% loss of shoot FW and DW, respectively.

The leaf area of both *Brassica* species was significantly reduced by all Zn concentrations (Fig. 2b). Although, higher doses of Zn treatment (between 150 and 300 μM) caused by ~8% slighter leaf growth inhibition in *B. napus* compared to *B. juncea*.

In the two *Brassica* species, excess zinc significantly decreased chlorophyll (chl) *a, b* and carotenoid contents, although, the effects were more pronounced in *B. juncea* (Supplementary Table 1). As the effect of 300 μM Zn, both total chlorophyll and carotenoid contents decreased more significantly in *B. juncea* leaves compared

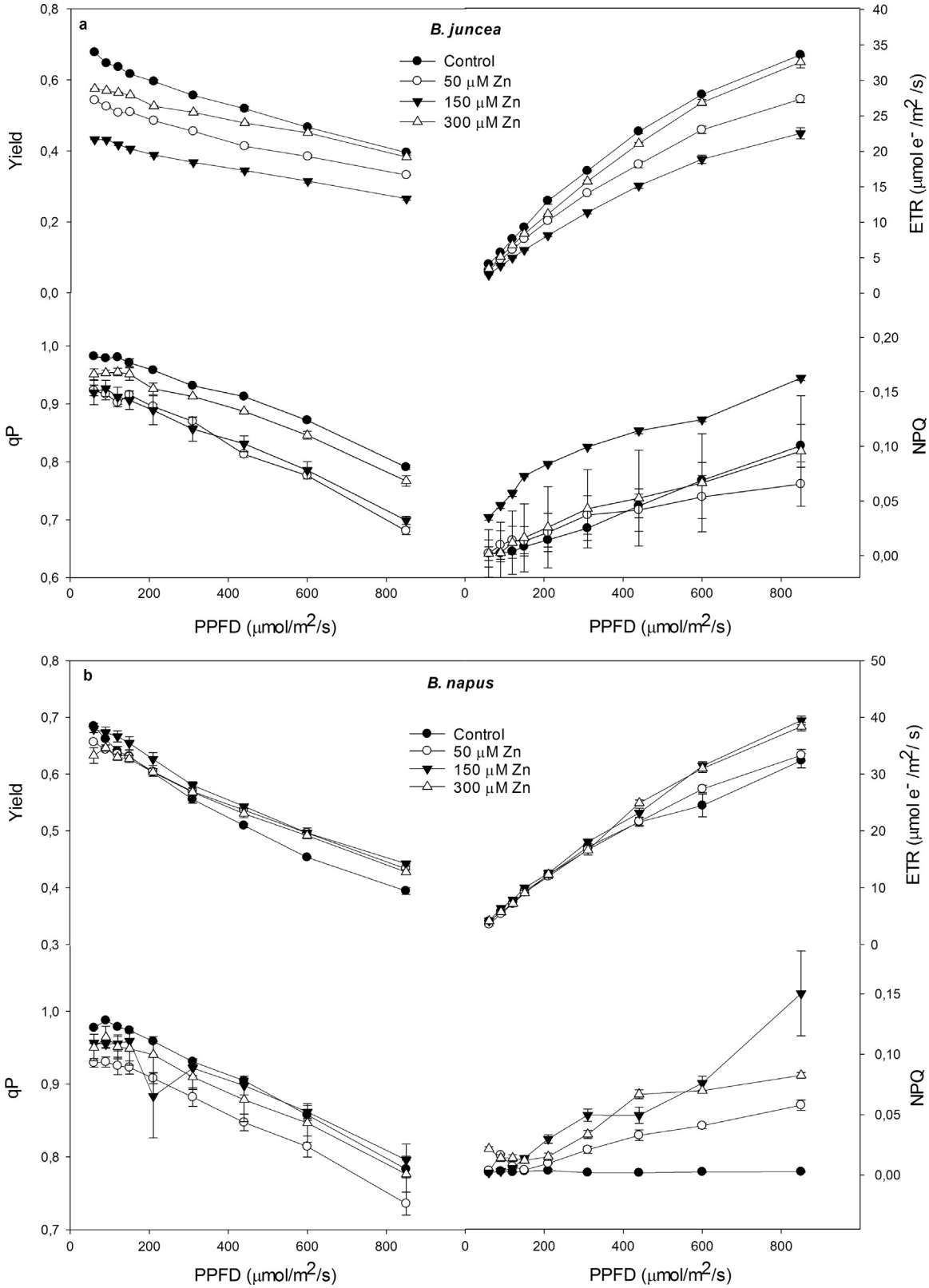


Fig. 3. Chlorophyll fluorescence parameters (Yield, ETR, qP, NPQ) of *B. juncea* (a) and *B. napus* (b) leaves after 14-days-long zinc exposure.

to *B. napus*. In *B. napus*, the rate of loss was greater in case of chl *b* compared to chl *a*, which resulted in the increment of chl *a*/*b* ratios suggesting that chl *b* pool is more sensitive to excess Zn than chl *a*. By Ebbs and Uchil (2008) two possible mechanisms were supposed for Zn-induced chlorophyll loss including the increased conversion of chl *b* to chl *a* contributing to the maintenance of the more important chl *a* pool under zinc stress. The other possible mechanism can be the metal-induced down-regulation of chl *a* oxygenase enzyme involved in chl *b* synthesis. Moreover, iron deficiency (see Supplementary Fig 1), or substitution of the central magnesium ion with Zn may also contribute to the observed Zn-triggered chlorophyll loss (Prasad and Strzalka, 1999). Chlorosis can be associated also with Mn deficiency (Last and Bean, 1991), which in our system can also be the reason of the chlorophyll diminution.

In Fig. 2c and d, Zn-triggered changes in leaf morphology and chlorotic symptoms can be seen. On the leaf blades of *B. juncea*, also necrotic lesions can be observed (marked by arrows in Fig. 2d) reflecting a serious damage induced by zinc in this species.

In order to get a more accurate view about the zinc-induced damage of the shoot system, chlorophyll fluorescence parameters were determined which provide a reliable method for assessing

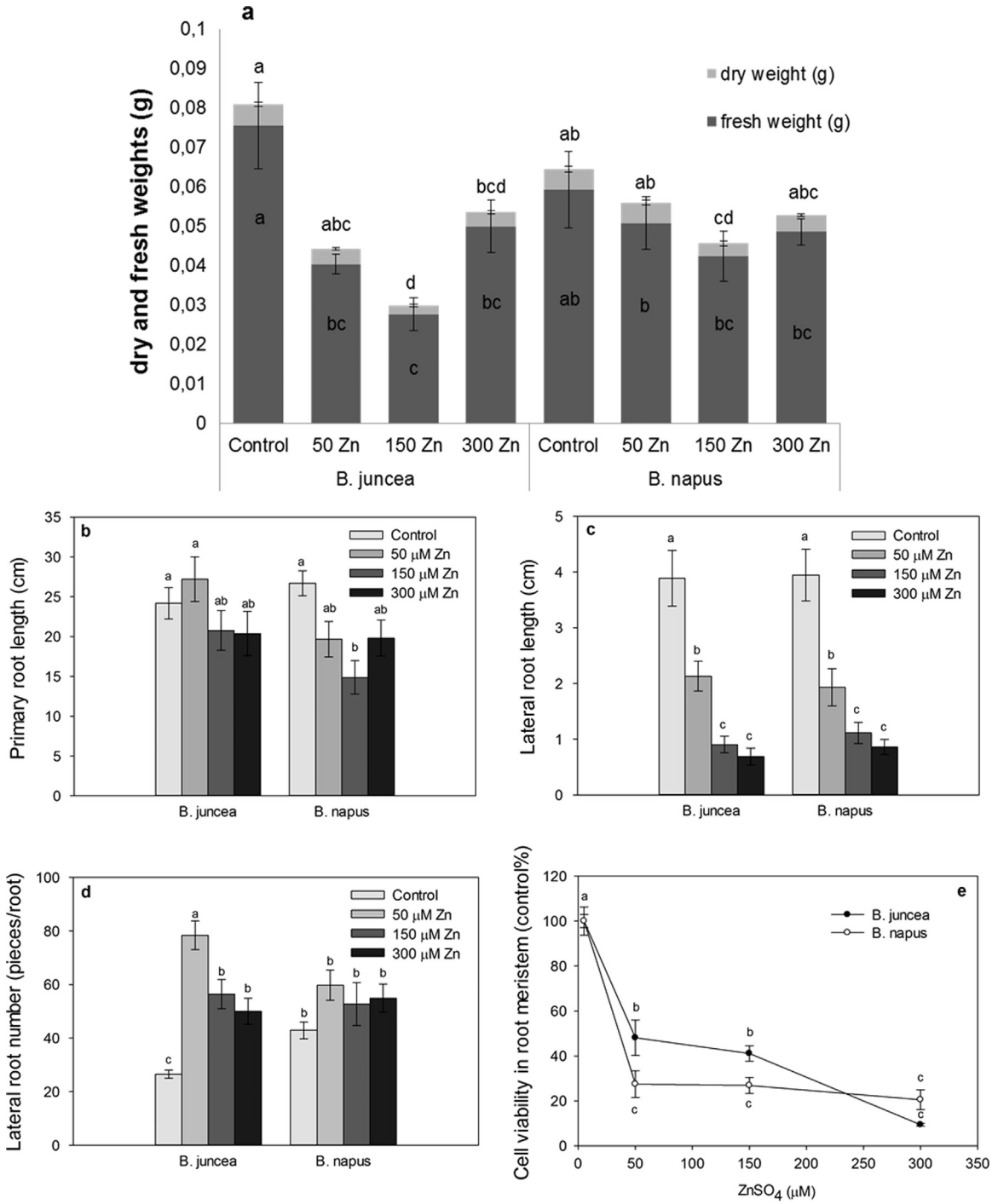


Fig. 4. (a) Dry and fresh weight (g) of the root system of *Brassica* species treated with 0, 50, 150 or 300 μM Zn. Length of the primary (b) and the lateral (c) roots and the number of lateral roots (d) in control and Zn-exposed *Brassica* species. (e) Viability of the root meristem cells (pixel intensity of fluorescein, control%) of control and Zn-treated *Brassica* species. Different letters indicate significant differences according to Duncan-test ($n = 10-20$, $P \leq 0.05$).

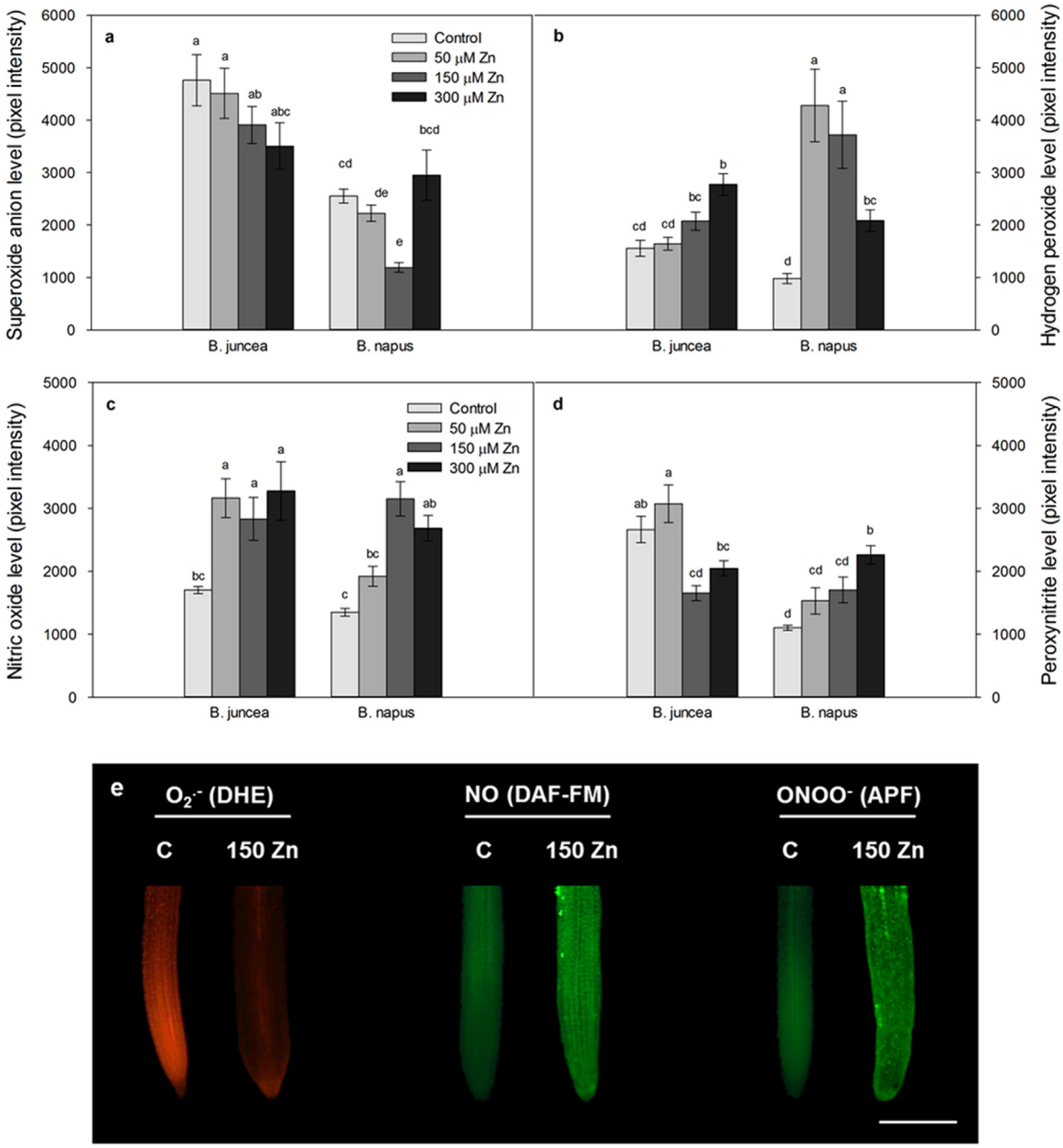


Fig. 5. The level of superoxide anion (pixel intensity of DHE, (a), hydrogen peroxide (pixel intensity of resorufin, (b), nitric oxide (pixel intensity of DAF FM, (c) and peroxynitrite (pixel intensity of APF, (d) in the root meristem of 0, 50, 150 or 300 μM Zn-exposed *B. juncea* and *B. napus*. Different letters indicate significant differences according to Duncan-test ($n=10-15$, $P \leq 0.05$). (e) Representative microscopic images of control and 150 μM Zn-treated *Brassica napus* root tips: DHE, DAF-FM, APF. Bar = 0.5 mm.

photosynthetic activity under stress conditions (Roháček et al., 2008). Exposure to excess Zn induced inhibition of photosynthesis especially in *B. juncea* (Fig. 3), while the effect was much slighter in *B. napus* leaves. Interestingly, the Yield, ETR and qP parameters of *B. juncea* were not affected by 300 and 50 μM Zn treatment remarkably, but they were the most seriously reduced by 150 μM Zn (Fig. 3a). The results indicate that excess Zn is an effective blocker of PSII function, especially in *B. juncea* leaves. Indeed, it has been demonstrated that the mechanism of action is the displacement of Mg by Zn at the water splitting site in photosystem II (Van Assche and Clijsters, 1986; Kupper et al., 1996). Moreover, Teige et al. (1990) suggested that the primary toxic action of Zn is the inhibition of ATP synthesis and therefore energy metabolism in plants. Another background mechanism of the photochemical activity loss during zinc toxicity can be the alteration of the inner structure and composition of the thylakoid membrane (Baszynski et al., 1988). In contrast to *B. juncea*, excess Zn did not result in obvious inhibition of the observed parameters (Yield, ETR, qP) in the leaves of *B. napus* (Fig. 3b). However, NPQ was found to increase as the effect of zinc exposure in both species. In *B. juncea*, only 150 μM Zn enhanced the NPQ parameter, while in *B. napus* all applied zinc concentrations increased the probability of dissipating the excess excitation energy via this alternative route. In our system, the photosynthetic activity well correlates with the iron deficiency-associated chlorosis, since zinc-treated *B. juncea* showed more intense biomass reduction, necrotic damages, chlorophyll loss and consequently more pronounced decrease in photosynthetic activity compared to *B. napus*. Therefore, we assume that the photosynthesis of *B. juncea* is more sensitive to zinc stress than that of *B. napus*.

In contrast to the shoot, the reducing effect of excess zinc on the fresh and dry weights of the root system proved to be

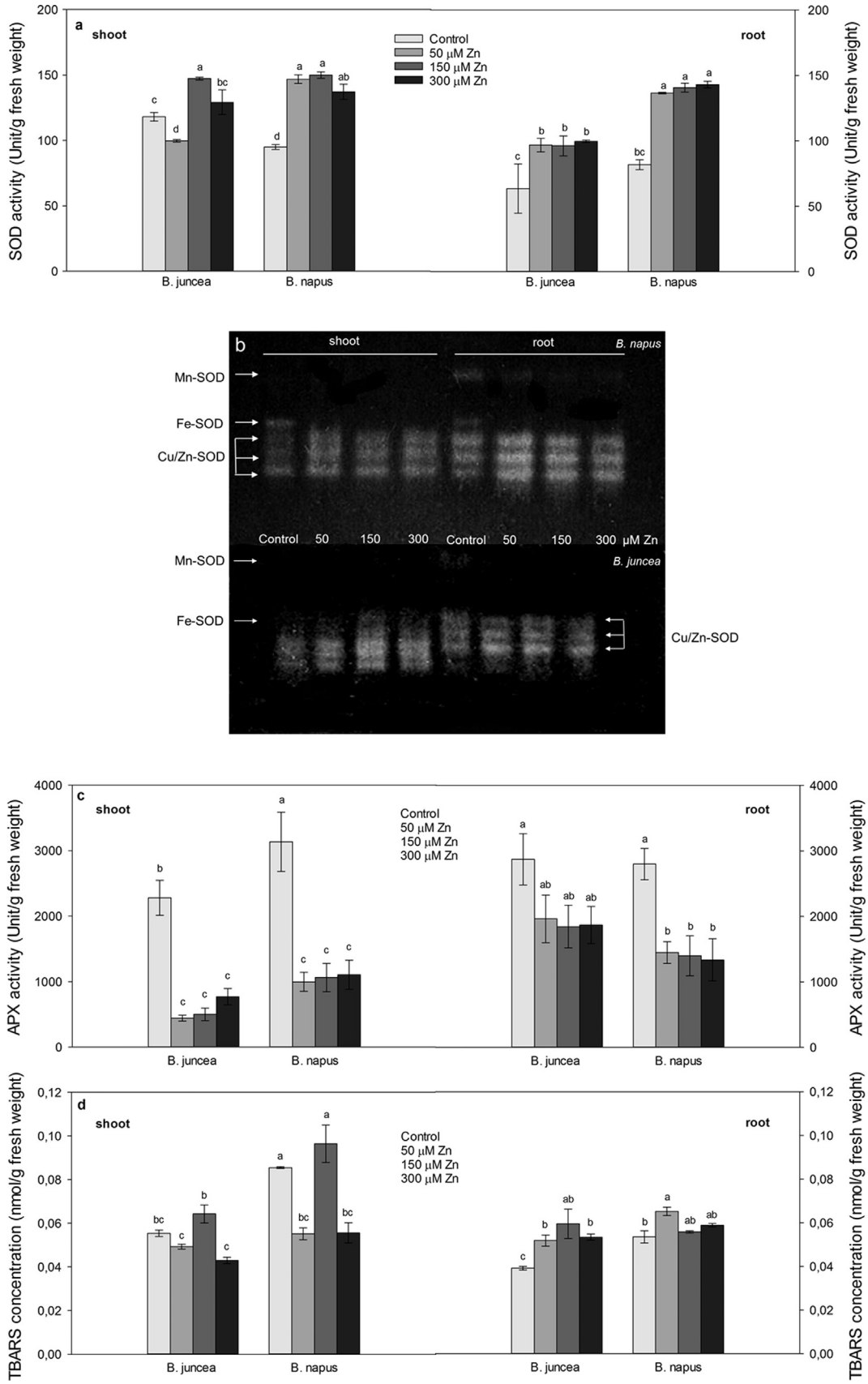


Fig. 6. Activity of SOD (unit/g fresh weight, a) enzyme and the analysis of SOD isoforms in the shoot and root of *B. napus* and *B. juncea* (b). SOD isoforms were separated by native-PAGE and stained by a photochemical method using 30 μg of protein per lane. Activity of APX (unit/g fresh weight, c) in the shoot and root system of *Brassica* species treated with 0, 50, 150 or 300 μM Zn. (d) Concentration of TBARS (nmol/g fresh weight) in the shoot and root of control and Zn-treated *Brassica* species. Different letters indicate significant differences according to Duncan-test ($n=6$, $P \leq 0.05$).

independent from the applied concentrations (Fig. 4a). In both species, the most serious diminution was observed in case of 150 μM $ZnSO_4$ treatment; although this effect was statistically significant only in *B. juncea*, despite that the root of this species accumulated smaller amount of zinc from the solution than that of *B. napus* (see Fig 1a). This suggests the greater zinc sensitivity of *B. juncea* compared to *B. napus*. During the detailed examination of the root architecture, some interesting differences were observed between the species. In *B. napus*, the elongation of the primary root was significantly inhibited by 150 μM Zn; however it was only slightly, but not significantly affected in *B. juncea* (Fig. 4b). This difference can be explained by the higher Zn accumulation of *B. napus* roots (see Fig. 1a). Regarding the lateral roots, excess zinc resulted in a remarkable and concentration-dependent shortening of them in both species (Fig. 4c), which refers to the higher sensitivity of the newly formed laterals compared to the primary root. Interestingly, zinc at all concentrations significantly increased the lateral root number of *B. juncea*, while in *B. napus* the effect proved to be much slighter (Fig. 4d). The accession of lateral root number induced by heavy metals was described as a symptom of stress-induced morphogenetic response (SIMR, Potters et al., 2009). Similarly to our results, LR-inducing effect of Zn in *Sesbania* species was reported by Yang et al. (2004). The changes in meristem cell viability showed correlation with the zinc-induced shortening of the PRs, which supports the fundamental role of meristem cell activity in root elongation. The viability loss was notable already in 50 and 150 μM Zn-exposed roots of the species, but in *B. napus* the viability reduction (Fig. 4e) as well as the PR shortening (Fig. 4b) proved to be more pronounced. Zinc-triggered cell death in the root system was proved, *inter alia*, in rice (Chang et al., 2005).

3.4. Excess zinc triggers changes in the ROS and RNS metabolism of the root system

Fluorescent microscopic techniques were applied for detecting the possible zinc-induced changes in ROS (superoxide radical and hydrogen peroxide) and RNS (nitric oxide and peroxynitrite) levels of the root system. During control circumstances all fluorophores showed higher fluorescence intensities in *B. juncea* roots than in those of *B. napus*. As the effect of zinc exposure, superoxide level slightly decreased in *B. napus*, in a concentration-dependent manner, while in the root tips of *B. napus* 150 μM zinc caused the most serious superoxide anion depletion (Fig. 5a). In both species the level of H_2O_2 was remarkably enhanced by zinc; although the accumulation was more intense in B. *juncea* roots (Fig. 5b). The highest H_2O_2 contents were detected in *B. napus* treated with 50 and 150 μM zinc. All zinc concentrations significantly increased the NO levels in *B. juncea* (Fig. 5c). In *B. napus*, the zinc-triggered NO generation proved to be slighter, but the 150 μM zinc exposure resulted in comparably high NO level. The possible mechanisms underlying Zn-induced NO formation may be diverse. According to Xu et al. (2010), Zn-triggered Fe-deficiency could lead to NO formation; although in our experiments, the Fe content of the root system did not decrease (see Supplementary Fig 1). Furthermore, in our earlier experiments, the activity of the major NO-producing enzyme, nitrate reductase, was not influenced by excess zinc in *Brassica* roots (Bartha et al., 2005). Instead, another possibility of NO production in this system is the transition metal-triggered decomposition of NO pools such as S-nitrosoglutathione (Smith and Dasgupta, 2000) but this hypothesis is needed to be confirmed in the future. Nitric oxide reacts with superoxide anion yielding peroxynitrite ($ONOO^-$), a powerful oxidative and nitrosative agent (Arasimowicz-Jelonek and Floryszak-Wieczorek, 2011). Regarding the peroxynitrite content, interestingly the higher zinc doses (150 and 300 μM) reduced it in *B. juncea* roots (Fig. 5d), which showed no correspondence to the observed changes in NO and superoxide levels (Fig. 5c). In the background of the zinc-induced peroxynitrite diminution, the activation of putative decomposition pathways (e.g. ascorbic acid, flavonoids, peroxyredoxin, and glutathione reductase) can be supposed (Arasimowicz-Jelonek and Floryszak-Wieczorek, 2011). In contrast, the peroxynitrite levels increased in *B. napus* roots (Fig. 5d), in a concentration-dependent manner; however no correlation of this with superoxide levels was found (Fig. 5a).

Zinc at all applied concentrations intensified the activity of SOD enzyme in the roots of the species; however the effects were not dependent on the zinc doses and the activation was more pronounced in *B. napus* (Fig. 6a). Within the shoot system, similar tendencies were observed. The different SOD isoforms were separated by native PAGE and five activity bands were identified in the organs of both species (Fig. 6b). In Fig. 6b, a representative SOD activity gel from *B. napus* is shown. The uppermost band represented a Mn-SOD isoform, which activity decreased as the effect of increasing Zn concentrations in the roots. The diminution of Mn-SOD activity can be explained by the reduced availability of manganese as previously showed in Supplementary Fig 1. The Fe-SOD isoform was only present in the control sample of *B. napus*; its activity was seriously reduced by the zinc treatments. The last three bands showed Cu/Zn-SODs, which showed a remarkable activation, especially in the roots, which was in correlation with the overall SOD activity (see Fig. 6a). The intensification of Cu/Zn-SODs may be the result of the increment of the Zn and Cu contents triggered by zinc exposure (see Supplementary Fig 1). Contrary to our results, significantly reduced total SOD and isoenzyme activities were observed in rapeseed; although younger plants were subjected to more severe zinc stress than in our system (Wang et al., 2009). The activity of APX decreased as the effect of zinc exposure in both organs of both species (Fig. 6c). Interestingly, the activity loss was more pronounced in the shoot system of the species (especially in *B. juncea*). These imply that the effect of zinc on antioxidant enzymes (at least SOD and APX) is dependent on the plant age, the duration and the intensity of stress treatment. The observed changes in the antioxidant enzyme activities could explain the alterations in the ROS levels, since the zinc-triggered activation of SOD and deactivation of APX can be responsible for the superoxide depletion and the H_2O_2 accumulation in the roots. Regarding the lipid peroxidation being a marker of oxidative stress, no obvious tendencies and intensification were observed in the shoot system of the species (Fig. 6d). Only in *B. juncea* roots, a significant increase in the amount of lipid peroxides (e.g. TBARS) could be determined as the effect of all applied zinc concentrations.

3.5. Excess zinc induces changes in the level and the pattern of protein tyrosine nitration

Using Western blot analysis, the presence of several 3-nitrotyrosine-positive protein bands were detected in the untreated samples (Fig. 7), which suggests that a part of the protein pool of the organs is nitrated even under control conditions. This raises the possibility that tyrosine nitration is a basal regulatory mechanism of protein activity. Similarly, a basal nitration state of proteins was evidenced in different plant species such as sunflower, *Citrus*, pea and pepper (Chaki et al., 2009, Begara-Morales et al., 2013, Corpas et al., 2013, Chaki et al., 2015). Moreover, the protein pool of the shoot of both *Brassica* was more nitrated compared to the root system, where the 3-nitrotyrosine-positive signals were much weaker indicating the organ specificity of protein tyrosine nitration. In preliminary experiments, we did not observe concentration-dependent effect of zinc on the level of tyrosine nitration, therefore based on other data 150 μM zinc was chosen for further analysis. In general, the pattern of protein

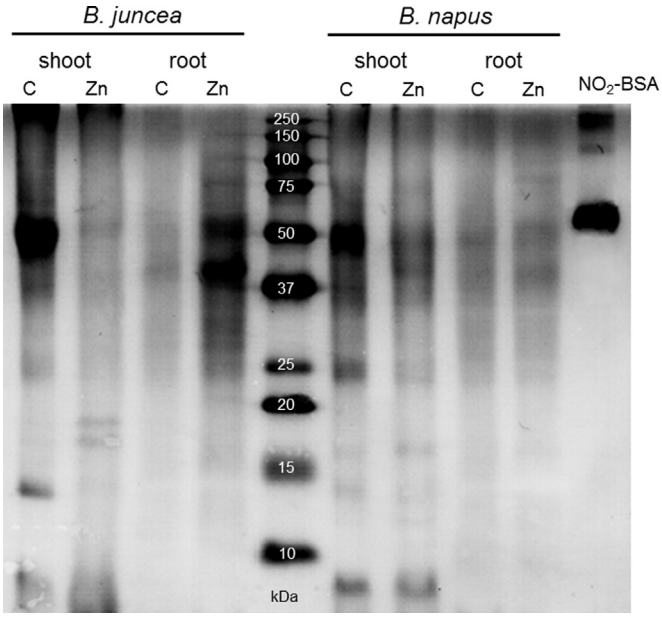


Fig. 7. Representative immunoblots showing protein tyrosine nitration in roots and shoots of *B. juncea* and *B. napus* plants under control conditions (C) and during 150 μM Zn exposure (Zn). Root and shoot samples were separated by SDS-PAGE and analysed on Western blotting with anti-nitrotyrosine antibody (1:2000). Commercial nitrated BSA (NO_2-BSA) was used as a positive control.

nitration was modified by zinc in the shoot system of the species, while a general strengthening of 3-nitrotyrosine-associated immunopositivity was observed in the root system of the zinc-exposed species. Based on this, we can assume that the proteome of the *Brassica* organs are differentially affected by zinc-triggered nitration. In the shoot of *B. juncea*, the nitration of the protein bands at 50, 37 and ~12 kDa decreased, while two new, immunopositive bands appeared between 15 and 20 kDa. In the shoot of zinc-exposed *B. napus,* the tyrosine nitration of protein bands at 50, 37, 25 and ~12 kDa weakened, while a slight intensification was observed in two other protein bands suggesting the modification of nitration pattern of the organ. In contrast to the shoot, the nitration level of the root proteome of both species intensified as the effect of 150 μM zinc; however, the response was much more intense in *B. juncea* roots. Similarly, the enhancement of the nitration levels was published, *inter alia*, in salt-stressed olive leaves, in cold-treated pea leaves or in arsenic-exposed *Arabidopsis* (rewieved by Corpas et al., 2013).

4. Conclusions

Among the two moderate Zn accumulator *Brassica* species, oilseed rape took up and translocated more zinc compared to *B. juncea*. Still the shoot of *B. napus* showed slighter zinc-induced damages (examined by growth and morphology parameters, pigment contents and photosynthetic activities), which were accompanied by the activation of antioxidants and the moderate alteration of protein nitration pattern. Based on the examined parameters (PR length, LR number, viability) the root system of *B. juncea* showed enhanced tolerance to zinc exposure compared to *B. napus*, and it was coupled with enhanced H_2O_2, NO levels and remarkably intensified protein nitration. 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). When we consider the zinc-induced changes of protein nitration in the shoot system, it can be assumed that the significant alteration of its pattern is coupled with enhanced zinc sensitivity, but the zinc-induced general intensification of protein nitration is rather attached to relative zinc endurance.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2015.12.006.

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