



## Zinc-induced root architectural changes of rhizotron-grown *B. napus* correlate with a differential nitro-oxidative response

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### ABSTRACT

Roots have a noteworthy plasticity: due to different stress conditions their architecture can change to favour seedling vigour and yield stability. The development of the root system is regulated by a complex and diverse signalling network, which besides hormonal factors, includes reactive oxygen (ROS) - and nitrogen species (RNS). The delicate balance of the endogenous signal system can be affected by various environmental stimuli, such as the excess of essential heavy metals, like zinc (Zn). Zn at low concentration, is able to induce the morphological and physiological adaptation of the root system, but in excess it exerts toxic effects on plants.

In this study the effect of a low, growth-inducing, and a high, growth inhibiting Zn concentrations on the early development of *Brassica napus* (L.) root architecture and the underlying nitro-oxidative mechanisms were studied in a soil-filled rhizotron system.

The growth-inhibiting Zn treatment resulted in elevated protein tyrosine nitration due to the imbalance in ROS and RNS homeostasis, however its pattern was not changed compared to the control. This nitro-oxidative stress was accompanied by serious changes in the cell wall composition and decrease in the cell proliferation and viability, due to the high Zn uptake and disturbed microelement homeostasis in the root tips. During the positive root growth response, a tyrosine nitration-pattern reorganisation was observed; there were no substantial changes in ROS and RNS balance and the viability and proliferation of the root tips' meristematic zone decreased to a lesser extent, as a result of a lower Zn uptake.

The obtained results suggest that Zn in different amounts triggers different root growth responses accompanied by distinct changes in the pattern and strength of tyrosine nitration, proposing that nitrosative processes have an important role in the stress-induced root growth responses.

### 1. Introduction

Heavy metal (HM) contamination of soils and water is an actual and growing challenge for the environment and for agriculture as well. This has partly originated from anthropogenic activities such as mining, waste disposal or agricultural processes for instance the excessive use of fertilisers or application of sewage [1], often causing higher Zn concentration than the typical 10–300 µg/g (ppm) in soils [2,3]. Zinc (Zn) is the second most abundant metal in living organisms [4], possessing a fundamental role in diverse physiological processes. As the only metal represented in all six enzyme classes [5], Zn is involved in

carbohydrate, lipid and nucleic acid metabolism and protein synthesis as well. Even though it is indispensable, it can be phytotoxic in amounts greater than the optimal. Zn as a non-redox active element is able to tightly bind to oxygen, nitrogen or sulphur atoms, inactivating enzymes by binding to their cysteine residues [6]. Zn is able to promote secondary oxidative stress by the replacement of essential metal ions in catalytic sites [7]. During Zn-induced oxidative stress, several reactive oxygen species (ROS), like superoxide anion (O<sub>2</sub><sup>-</sup>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), and hydroxyl radicals (OH) are commonly generated [8,9,83]. In order to ensure the plants' survival, the level of ROS has to be strictly regulated by a complex mechanism [10], including numerous

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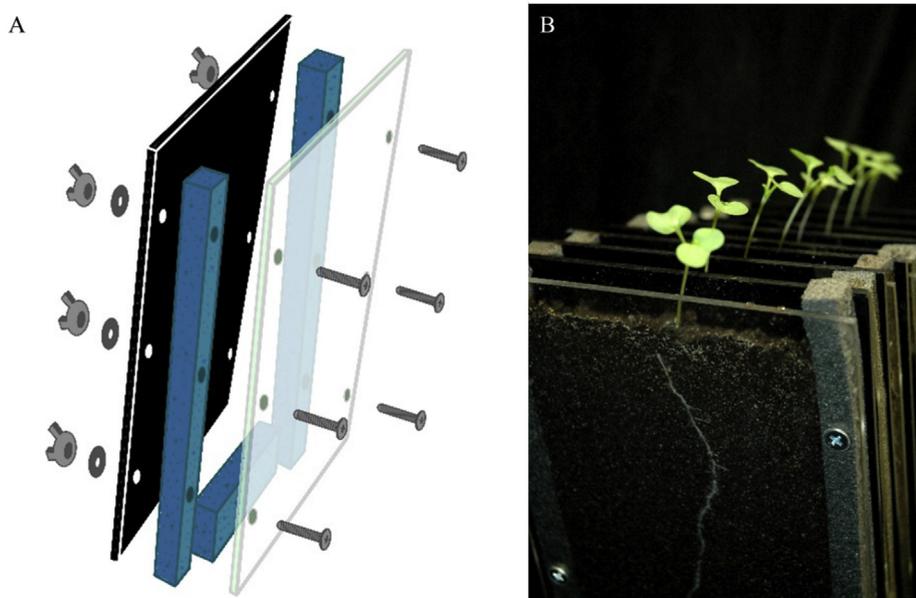


Fig. 1. Rhizotron design (A) and growing *Brassica napus* seedlings in soil-filled rhizotrons (B).

enzymatic antioxidants 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), or non-enzymatic antioxidants like ascorbate or glutathione.

In addition to ROS, reactive nitrogen species (RNS) are also being formed as the consequence of many different environmental stresses. The term RNS refer to the family of nitric oxide (NO) and associated molecules, including peroxynitrite ( $\text{ONOO}^-$ ) and S-nitrosoglutathione (GSNO), [11]. Nitrosative stress, analogue to oxidative stress is the consequence of the accumulation of the above-mentioned molecules in the plant cells, can be caused by numerous environmental factors [12,13].

The metabolisms of ROS and RNS are connected at several points. The concept of nitro-oxidative stress has only recently become the subject of research in the field of plant biology [14]. A typical example of ROS-RNS crosstalk is the reaction of  $\text{O}_2^-$  and NO resulting in the formation of  $\text{ONOO}^-$ , which is accountable for post-translational modification protein tyrosine nitration, the covalent modification on specific tyrosines in proteins forming 3-nitrotyrosine [15]. The addition of the nitro group to one of the ortho carbons in the aromatic ring of tyrosine residues [17] results in steric and electronic perturbations, modifying the tyrosine's ability to keep the proper conformation of the proteins or to function in electron transfer reactions [18]. Tyrosine nitration might affect the function of the proteins in different ways: the most common outcome is the loss of the protein's function, but rarely gain of function or the lack of effect has also been reported [15,19,20]. Moreover, tyrosine nitration is furthermore able to disturb signal transduction pathways by the inhibition of tyrosine phosphorylation [21].

Due to nitro-oxidative stress and disturbances in macro- and microelement homeostasis [8], excess Zn inhibits seed germination and plant growth [22,23] including root development [24]. HMs in high concentration lead to growth inhibition due to their phytotoxic effect by altering the most important plant physiological and metabolic processes [25], while on the other hand at low concentrations they are able to persuade the morphological and physiological adaptation of the root system called stress-induced morphogenic response (SIMR). SIMR is a special mixture of inhibition of primary root growth and induction of lateral root development, resulting in a shallower but horizontally more extensive root architecture, which most likely provides an enhanced stress tolerance [26,27]. A protection machinery in contradiction of

enhanced HM concentrations is the modification of the cell wall in the root by the addition of e.g. callose or pectin. This process can assist the survival of the plant by restraining the uptake and translocation of HMs and by inhibiting the outflow of nutrients and assimilates [28,29], and at the same time cell wall alterations modify root growth processes as well.

Tracking the growth of the root system in soil can be challenging, however number of research apply rhizotrons to *in situ* observe root system architecture of e.g. maize [30], trees [31], *Arabidopsis* [16] or *Brassica napus* under phosphorus deficiency [32]. Rhizotrons may vary in size, depending on the goal of the experiments and the investigated plant species, but in general their main feature is a transparent wall ensuring the *in situ* monitoring of the development of plants' root system. In the present study, a 15 cm wide and 30 cm tall rhizotron system was developed, allowing the observation the early development of *Brassica napus* root system.

Contaminants, like Zn are able to change interactions between soil organisms [34], hereby investigation of soil properties like enzyme activity can provide a more complete understanding of the effect of HM stress on plant-soil system [35]. There are both examples of decreased [36] and increased [37,87] enzyme functions due to contamination with different HMs, suggesting that soil microbial communities might be able to react differently to HM stress.

The crops' responses in their early developmental stage basically determine their subsequent development, thus studying of the zinc-induced changes in root architecture and the underlying mechanisms have a great significance. In a previous study we determined that *B. napus* is sensitive to Zn stress in a hydroponic system [38], but no experiments were conducted in the topic in a near-natural (soil filled rhizotron) setup. Therefore, our goal was to compare growth-inducing and growth-inhibiting Zn concentrations in soil for *Brassica napus*, and to determine whether if the nitro-oxidative signalling network is involved in the development of these different growth responses.

## 2. Materials and methods

### 2.1. Rhizotron system

Custom-made plexi panels were assembled into 15 cm wide, 30 cm tall and 1.6 cm thick rhizotrons, using polifoam sheets and screws with wing nuts. The front panel is made of 3 mm thick, anti-glare, 100%

transparent plastic, while the back panel is a 3 mm thick non-transparent black sheet; the thickness of the soil layer inside the rhizotron was 1 cm (Fig. 1). The rhizotrons were filled with Klasmann Potgrond P blocking substrate (100% frozen through black peat with a fine structure of maximum 8 mm size, pH 6.0; 210 mg N/l; 240 mg P<sub>2</sub>O<sub>5</sub>/l, 270 mg/l K<sub>2</sub>O, 60.21 mg/kg (ppm) Zn) mixed with 20% sand; the initial water content was set to 70%. Based on preliminary experiments, 10 and 500 ppm Zn supplementation were chosen as acclimation-causing (growth-inducing) and growth-inhibiting concentrations, respectively; Zn supplementation was homogeneously distributed in the mixture by manual mixing.

## 2.2. Plant material and growing conditions

*Brassica napus* L. (GK Gabriella; oilseed rape or rapeseed) seeds provided by the Cereal Research Non-Profit Ltd. (Szeged, Hungary) were pre-germinated for 24 h at 26 °C and germinated seeds were transferred to the soil surface of the pre-filled rhizotrons (one seed per rhizotron, Fig. 1B). During the first 48 h after the sowing, the seedlings were covered with transparent plastic foil to provide optimal humidity, then the growing plants were supplemented with 10 mL distilled water on every second day. Seedlings were cultivated in greenhouse at photon flux density of 150 μmol m<sup>-2</sup> s<sup>-1</sup> (12/12 h light/dark cycle) at a relative humidity of 55–60% and 25 ± 2 °C for 10 days, then the rhizotrons were scanned, disassembled and the roots were cleaned for further examination. In some cases, daily scanning was also performed to obtain images for the representation of the growth dynamics of the root system (videoS1 Supplementary Video 1).

Supplementary video related to this article can be found at <https://doi.org/10.1016/j.niox.2019.06.003>.

## 2.3. Morphological measurements

Scanned images of the rhizotrons were analysed using Fiji software (<http://fiji.sc/Fiji>; [39]). The length of the primary root (PR; mm) was measured; the number of visible lateral roots were counted (LR; laterals per root) and their length (mm) and angle included with the vertical direction (degrees) were also measured. These data were acquired from eight to ten separate generations, in each generation eight plants were examined (n = 8).

## 2.4. Element content analysis

The concentrations of microelements were measured by inductively-coupled plasma mass spectrometry (ICP-MS, Thermo Scientific XSeries II, Asheville, USA) according to Ref. [40]. Values of Zn and other microelement concentrations (Fe, Cu, Mn, Ni, Cr, Co and Mo) are given in μg/g dry weight (DW). Bioaccumulation factor (BAF, Zn concentration in the shoot/Zn concentration in the soil) and translocation factor (TF, Zn concentration in the shoot/Zn concentration in the root) was calculated according to Ref. [42]. This analysis was carried out twice with three samples each (n = 3).

## 2.5. Microscopic determination of Zn distribution, callose and pectin deposition, lipid peroxidation, viability and DNA replication capability in the root tissues

For the detection of Zn uptake, root tips were washed in PBS buffer (137 mM NaCl, 2.68 mM KCl, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.47 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4), and then dyed with 25 μM Zinquin (ethyl (2-methyl-8-*p*-toluenesulphonamido-6-quinolyloxy)acetate) in PBS for 1 h at room temperature in darkness as described by Ref. [43].

Callose content of the root tips' cell walls was visualised by using aniline blue staining according to Ref. [38]. Roots tips were dyed in aniline blue solution (0.1%, w/v in 1 M glycine) for 5 min, then replaced by distilled water prior to microscopic analysis.

Cell wall pectin content was detected by using 0.05% (w/v) ruthenium red (RR) solution prepared with distilled water, according to Ref. [44].

Viability of meristematic cells in the root was determined by fluorescein diacetate (FDA) staining, according to Ref. [45]; roots were dyed with 10 μM staining solution prepared in 10 mM MES (4-morpholineethanesulfonic acid)/ 50 mM KCl buffer (pH 6.15). FDA is a cell membrane-permeant esterase-substrate, which is widely used as a viability probe, which measures enzymatic (intracellular esterase) activity (it is required to activate its fluorescence) and membrane integrity (it is required for the retention of the fluorescent product) [46].

To evaluate DNA replication prior to cell proliferation in root tips 5-ethynyl-2'-deoxyuridine (EdU) was used as described by Ref. [47] with slight modifications. Root segments were incubated in 20 μM EdU solution (prepared in PBS) in darkness for 2 h followed by incubation in detergent buffer (PBS buffer pH 7.4 containing 4% formaldehyde and 0,5% Triton X-100). Samples were washed three times with PBS and incubated for 30 min in reaction buffer (40 mM ascorbate, 4.2 mM CuSO<sub>4</sub> and 3.6 μM Alexa Fluor 488 azide in PBS). To determine the number of cells in which EdU incorporation has occurred in the apical meristem, fluorescent cells were counted within circles of 50 μm radii. These measurements were carried out twice with 10–15 samples each (n = 10–15).

## 2.6. Detection of ROS and RNS

Fluorescence consistent with superoxide anion in the root tips was detected by using dihydroethidium (DHE) (30 min incubation in darkness at 37 °C with 10 μM dye solution followed by two washing with 10 mM Tris/HCl, pH 7.4) [48]. Fluorescence consistent with hydrogen peroxide was detected by the incubation of root tips in 50 μM Ampliflu™ (10-acetyl-3,7-dihydroxyphenoxazine, ADHP or Amplex Red) solution (prepared in 50 mM sodium-phosphate buffer, pH 7.5), according to Ref. [40].

Fluorescence consistent with NO in *Brassica* root tips were determined by 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA), by incubation in 10 μM dye solution prepared in 10 mM Tris/HCl buffer, (pH 7.4) for 30 min in darkness at room temperature [49]. Fluorescence consistent with peroxyntirite was visualised with 10 μM dihydrorhodamine 123 (DHR) prepared in Tris-HCl buffer. After 30 min of incubation, root tips were washed with buffer two times [50].

These measurements were carried out twice with 10–15 samples each (n = 10–15). Suppl. fig. 5 shows positive and negative controls for the applied fluorescent dyes.

## 2.7. Immunofluorescent microscopic detection of 3-nitrotyrosine in root tissues

For immunofluorescent staining, small pieces of root samples derived from the root tips were fixed in 4% (w/v) paraformaldehyde according to Ref. [51]. Following fixation, root samples were rinsed with distilled water and fixed in 5% agar (bacterial [52]; with modifications). Then 100 μm thick longitudinal sections were made using a vibratome (VT 1000S, Leica).

Immunodetection of 3-nitrotyrosine was carried out according to Ref. [53] as described by Ref. [54].

Immunofluorescent detections were carried out on two separate plant generations with 8 plants examined in each (n = 8).

## 2.8. Acquisition and processing of microscopic images

*Brassica* root samples labelled with different fluorescent dyes were examined under a Zeiss Axiovert 200 M inverted microscope (Carl Zeiss, Jena, Germany). Filter set 9 (exc.: 450–490 nm, em.: 515–∞ nm) was used for DHE; filter set 10 (exc.: 450–490, em.: 515–565 nm) was applied for DAF-FM, DHR, FDA and FITC; filter set 20HE (exc.: 546/12,

em.: 607/80) was used in case of AmplexRed and filter set 49 (exc.: 365 nm, em.: 445/50 nm) was utilised with aniline blue, EdU and Zinquin staining.

Fluorescence intensities (pixel intensity, consistent with the amount of the detected molecule) in the meristematic zone were measured on the acquired images using Axiovision Rel. 4.8 software within circles of 50  $\mu\text{m}$  radii.

### 2.9. Determination of soil catalase activity

Activity of catalase in soil was measured by a titrimetric method according to Ref. [55]. 2 g soil from each rhizotron was added to a mixture of 40 mL distilled water and 5 mL 0.3%  $\text{H}_2\text{O}_2$ . After 20 min of shaking, 5 mL of 1.5 M  $\text{H}_2\text{SO}_4$  was added and the suspension was filtered, then titrated with 0.02 M  $\text{KMnO}_4$ . Catalase activity (CAT) was expressed as  $\mu\text{mol H}_2\text{O}_2/\text{g dry soil weight}/\text{min}$  calculated from the reacted amount of 0.02 M  $\text{KMnO}_4$ . Soil samples without  $\text{H}_2\text{O}_2$  addition were used as blanks.

This measurement was carried out on two separate generations with 3 examined soil sample each ( $n = 3$ ).

### 2.10. Measurement of root SOD activity and SOD isoform staining on native-PAGE

SOD (EC 1.15.1.1) activity of *Brassica napus* roots was determined according to Ref. [56], as described by Ref. [38]; enzyme activity is expressed in Unit/g fresh weight. SOD isoforms were detected in gels by the modified method of [57] as described by Ref. [38].

These experiments were carried out on two separate plant generations with three samples examined each ( $n = 3$ ).

### 2.11. NADPH-oxidase (NOX) activity of the roots on native-PAGE

NOX activity was examined on 10% native polyacrylamide gels by the NBT reduction method of [58] with slight modifications published by Ref. [54]. 25  $\mu\text{l}$  of protein extracts were loaded in each well.

These experiments were carried out on two separate plant generations with three samples examined each ( $n = 3$ ).

### 2.12. GSNOR activity on native-PAGE

GSNOR activity was visualized using a slightly modified method of [59] and is described in detail by Ref. [54]. 50  $\mu\text{l}$  of protein extracts were loaded in each well.

These experiments were carried out on two separate plant generations with three samples examined each ( $n = 3$ ).

### 2.13. SDS-PAGE and western blotting for NO-Tyr and GSNOR

Protein extracts of *Brassica napus* root tissues were prepared as described in Ref. [54]; protein concentration was determined using the [60] assay with bovine serum albumin as a standard. 20  $\mu\text{l}$  of root protein extracts per lane were subjected to sodium dodecyl sulphate-PAGE (SDS-PAGE) on 12% acrylamide gels, followed by procedures described by Ref. [54].

Immunoassay for GSNOR enzyme was performed using a polyclonal primary antibody from rabbit diluted 1:2000 purchased from Agrisera (AS09 647). As secondary antibody affinity-isolated goat anti-rabbit IgG-alkaline phosphatase secondary antibody (Sigma-Aldrich, cat. No. A3687) was used at a dilution of 1:10000, and bands were visualized by using the NBT/BCIP (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) reaction.

Western blot was applied to three separate protein extracts from different plant generations, multiple times per extract, giving a total of six blotted membranes ( $n = 3$ ).

Protein bands of SOD, NOX, GSNOR enzyme and nitrated proteins

were quantified by Gelquant software provided by [biochemlabsolutions.com](http://biochemlabsolutions.com).

### 2.14. Statistical analysis

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 ( $*P \leq 0.05$ ,  $**P \leq 0.01$ ,  $***P \leq 0.001$ ).

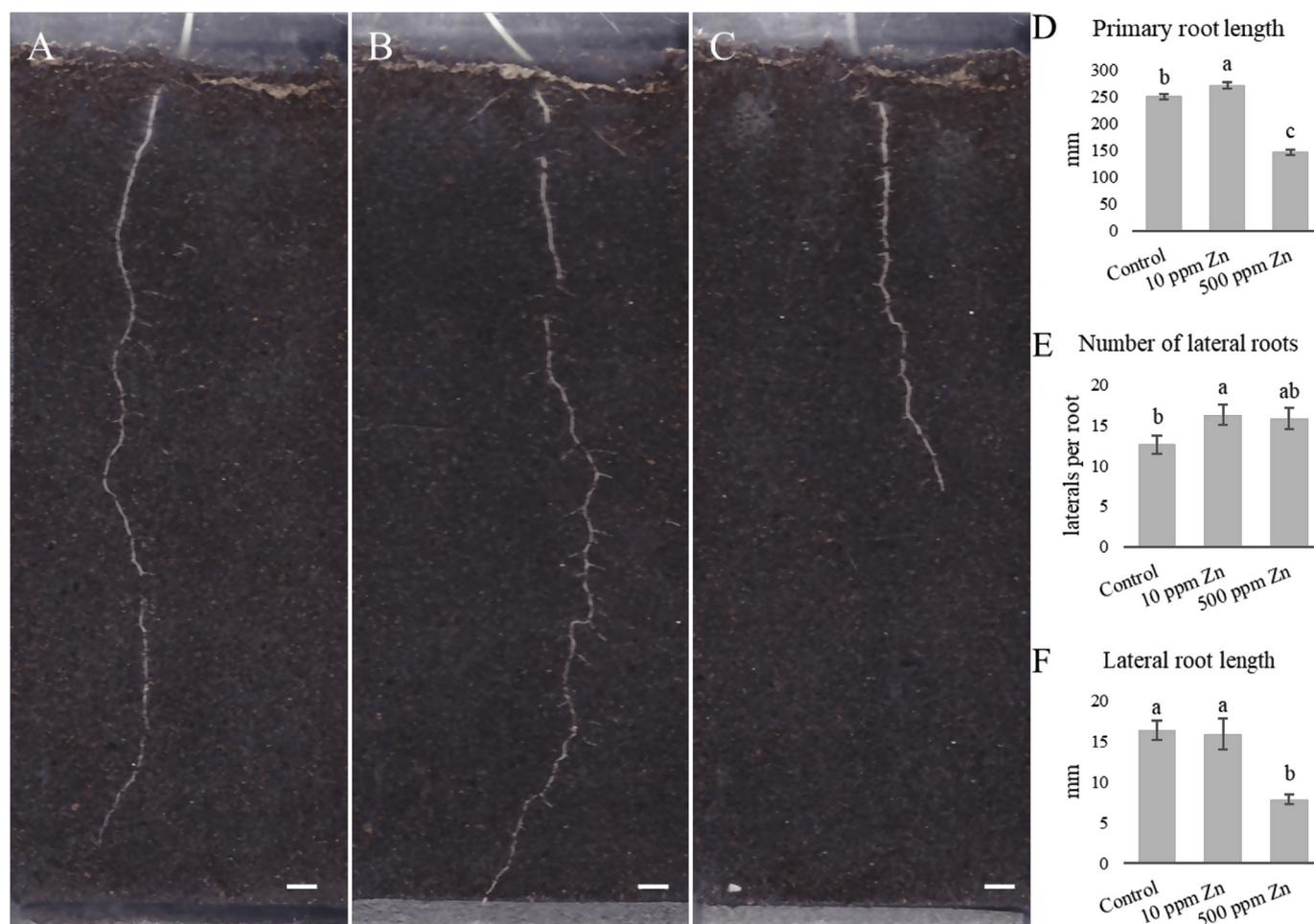
## 3. Results and discussion

### 3.1. Zn uptake-induced changes in root architecture, root cell wall composition and microelement homeostasis

As previously stated, control soil contained 60 ppm total Zn, thus 10 and 500 ppm Zn supplementation resulted in 70 (within the typical 10–300 ppm range) and 560 ppm (over the typical range) total soil Zn content, respectively. Zn exists in five distinct pools in soils such as water soluble, exchangeable, adsorbed, chelated or complexes of Zn [61]; however the investigation of the form and bioavailability of the total Zn in the soil were not our aim, thus were not examined. Throughout the article control refers to soil containing 60 ppm Zn, as provided by the manufacturer.

The rhizotron system allows the easy monitoring of the development of the root system architecture (RSA) (Fig. 2A). Compared to the control, both Zn supplementations caused significant changes in the RSA (for the dynamics of RSA development see videoS1 Supplementary Video 1). Mild Zn treatment (10 ppm supplementation) induced root growth in terms of the length of the primary root (107%) and number of lateral roots (129%) (Fig. 2B and C), while the length of lateral roots remained similar to the control (Fig. 2D). On the other hand, high Zn concentration (500 ppm supplementation) inhibited primary and lateral root elongation (58 and 48%, respectively) (Fig. 2B, D), while the number of lateral roots (similar to the 10 ppm supplementation) were higher than in the control (125%) (Fig. 2C). It can be noted, that due to the significant shortening of the primary root under 500 ppm Zn supplementation, lateral root density increased noticeably compared to control conditions (LR/cm; control: 0.5, 500 ppm Zn: 1.1). In a previous, hydroponic study, lateral root number of *B. juncea* and *B. napus* was also increased by Zn excess [62]; and this phenomenon is also a known symptom of SIMR [63]. Moreover, in *Sesbania* species, Zn also induced lateral root formation [84]. Interestingly, the angle of lateral roots relative to the vertical direction also changed significantly due to Zn supplementation, but the response was different depending on Zn concentrations: addition of 10 ppm Zn induced a more horizontal lateral root growth (control: 65°, 10 ppm Zn 68°), while 500 ppm Zn supplementation led to a more vertical (60°) lateral root orientation (Suppl. fig. 1). This contrasting response corroborate the opposite effect of the two applied Zn treatment: low Zn-induced growth induction is accompanied by a more horizontal root system (acclimation), while the growth-inhibiting Zn concentration caused the lateral roots to grow more to the vertical direction, possibly as compensational reaction. According to our hypothesis, since the PR growth is inhibited by 500 ppm Zn supplementation, the LRs are aiming to the deeper zones, while addition of 10 ppm Zn did not inhibit PR growth and the LRs are expanding laterally, since they are able to acclimatise to the mild Zn treatment. Many studies discuss the regulation of root angle determination by a complex series of internal and external factors [64], however the existence or background of Zn-induced changes in the lateral root angle is yet to be discovered.

According to the obtained RSA data, the two applied Zn concentrations causes two distinctly different responses: the effect of 10 ppm Zn supplementation has an overall positive consequence, while 500 ppm Zn supplementation inhibits longitudinal growth (PR and LR)



**Fig. 2.** (ABC) Representative images of the effect of Zn on the root system architecture of 10-days-old *B. napus*. (A – control, B – 10 ppm Zn supplementation, C – 500 ppm Zn supplementation; bar = 1 cm). Length of the primary root (D) and the effect of Zn on the number (E) and length (F) of lateral roots. Different letters indicate significant differences according to Duncan-test ( $n = 8$ ,  $P < 0.05$ ).

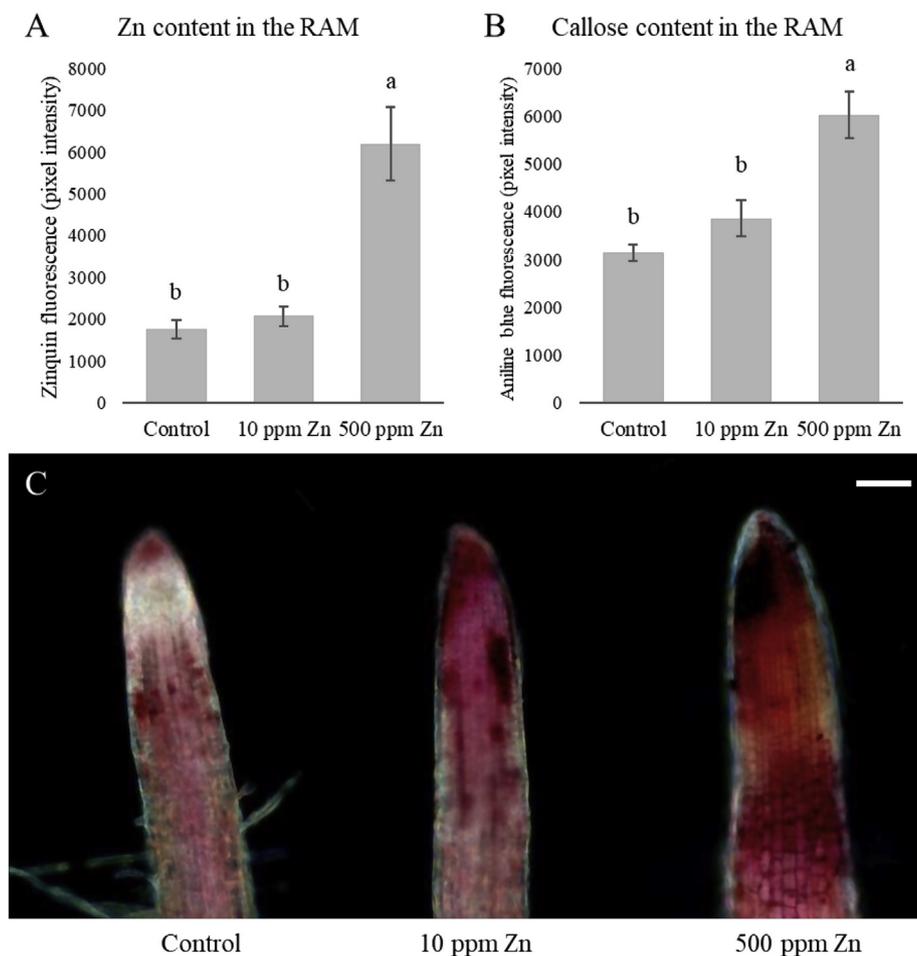
and induces branching process at the same time. It has to be also noted that though 500 ppm Zn treatment caused responses that could even meet the requirements of SIMR [63], however its negative, growth inhibiting effects are more pronounced.

Microscopic analysis of the Zn content in the root apical meristem (RAM) revealed that the meristematic zones of plants grown in the 10 ppm Zn supplemented soil did not accumulate significantly more Zn than the control, while the addition of 500 ppm Zn caused significant Zn uptake (3.5-fold increase) in their root apical meristems (Fig. 3A). Root cell wall modifications can indicate and prevent heavy metal uptake. Callose content of the root tips shows a similar tendency to the Zn contents, namely only the high Zn concentration caused significant (almost two-fold, compared to the control) callose deposition (Fig. 3B). Excess Zn reportedly caused significant callose deposition in e.g. bean [65] and *B. juncea* and *B. napus* [38]. The high amount of deposited callose might contribute to growth inhibition, since it decreases cell wall loosening and inhibits symplastic transport [66,85]. Callose is not permeable to metal ions [67], thus prevents Zn to enter the cells. On the other hand, both Zn treatments increased pectin content in the root tips, providing a possible explanation how 10 ppm Zn-treated root meristem is able to exclude Zn. Although, in case of 500 ppm Zn addition, RAM showed more pronounced pectin staining compared to 10 ppm Zn treatment (Fig. 3C). Pectin is able to bind HMs in the cell walls [68] and the observed pectin accumulation due to the 10 ppm Zn treatment could be enough to exclude Zn but probably it was not sufficient against 500 ppm Zn supplementation. The observed changes in pectin and callose content could complement each other: the increase of pectin

may bind Zn in the cell wall, and the deposited callose immobilizes it in the cell wall and ensures that it does not enter the cytoplasm. The latter can contribute to the above discussed growth inhibition as well.

According to the ICP-MS measurements Zn content of the whole root system was significantly increased by both Zn treatments in a concentration dependent manner (2.8 and 175-fold, compared to the control) (Table 1). Additionally, Zn was translocated to the shoot in a concentration-dependent manner. Bioaccumulation factor (BAF, shoot/soil concentration ratio) is a suitable tool to assess the plants' metal accumulation potential hence their phytoremediation ability [69]. The BAF under control circumstances was 3.19, while 10 and 500 ppm Zn treatment enhanced it to 5.32 and 4.77, respectively, proving that *B. napus* is a moderate Zn accumulator species, since in excluder plants this value is below 1 [70]. Also, in our previous works, similar Zn accumulation tendencies were observed [38,62]. Translocation factor (TF), as Zn concentration ratio of plant shoots to roots can also be used to evaluate a species' phytoremediation potential [71]. Contrary to BAF values, TF proportionally decreased by the increasing external Zn concentration (0.74; 0.51 and 0.05, respectively), indicating that though rapeseed is a moderate accumulator, its translocation capacity is low.

Zn supplementation of the soil also modified the microelement homeostasis of *B. napus* roots. Iron (Fe), cobalt (Co) and molybdenum (Mo) content of the roots decreased in a concentration-dependent manner, while the decline of copper (Cu), nickel (Ni), chromium (Cr) concentrations was not proportional to the increasing Zn concentration (Table 1). On the other hand, manganese (Mn) content increased due to



**Fig. 3.** Zinc (A) and callose (B) content in the root apical meristem of 10 days-old *Brassica napus* grown in soil-filled rhizotrons in the presence of optimal (control, 60 ppm Zn) or supraoptimal (10 and 500 ppm supplementation) Zn levels. Different letters indicate significant differences according to Duncan-test ( $n = 10-15$ ,  $P < 0.05$ ). (C) Representative image showing the pectin-associated pink colorization in the *Brassica napus* root tips (bar = 100  $\mu\text{m}$ ).

**Table 1**

(A) Zn content of the *B. napus* organs and BAF/TF values. (B) Microelement content of the roots ( $\mu\text{g/g DW}$ ). Different letters indicate significant differences according to Duncan-test ( $n = 3$ ,  $P < 0.05$ ).

A	Control	10 ppm Zn	500 ppm Zn
Zn root ( $\mu\text{g/g DW}$ )	258.7 $\pm$ 3.87 <sup>c</sup>	723.3 $\pm$ 22.15 <sup>b</sup>	45300 $\pm$ 109.6 <sup>a</sup>
Zn shoot ( $\mu\text{g/g DW}$ )	191.8 $\pm$ 0.84 <sup>c</sup>	373 $\pm$ 2.98 <sup>b</sup>	2672 $\pm$ 32.57 <sup>a</sup>
BAF Zn	3.19	5.32	4.77
TF Zn	0.74	0.51	0.05

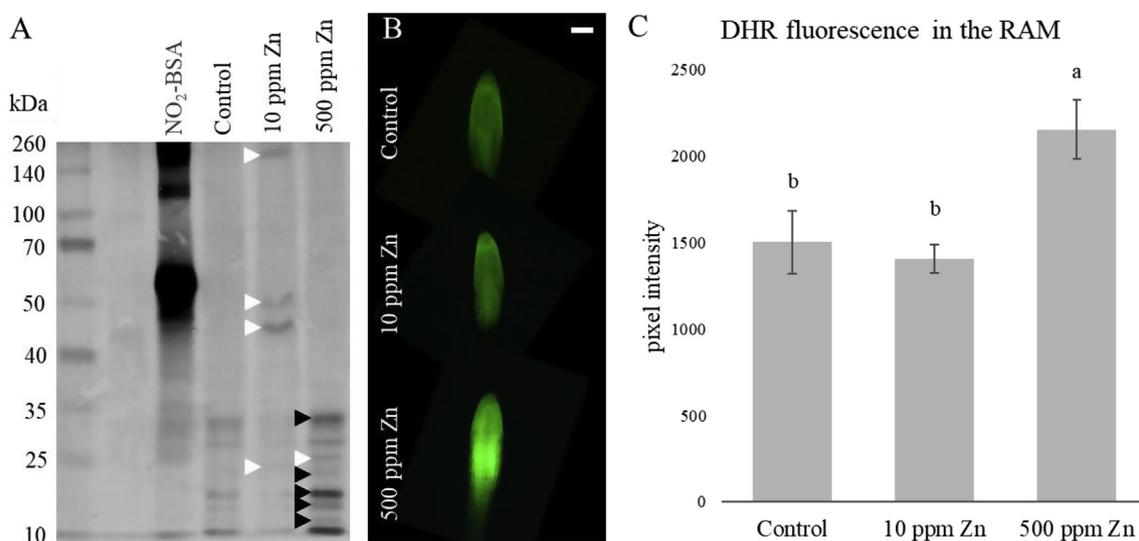
B	Control	10 ppm Zn	500 ppm Zn
Fe root ( $\mu\text{g/g DW}$ )	744.9 $\pm$ 4.81 <sup>a</sup>	515 $\pm$ 4.02 <sup>b</sup>	284.9 $\pm$ 2.48 <sup>c</sup>
Cu root ( $\mu\text{g/g DW}$ )	56.01 $\pm$ 0.95 <sup>a</sup>	9.06 $\pm$ 0.44 <sup>b</sup>	9.32 $\pm$ 0.05 <sup>b</sup>
Mn root ( $\mu\text{g/g DW}$ )	233.5 $\pm$ 1.73 <sup>c</sup>	240.8 $\pm$ 2.06 <sup>b</sup>	322.9 $\pm$ 1.25 <sup>a</sup>
Ni root ( $\mu\text{g/g DW}$ )	267.3 $\pm$ 1.33 <sup>a</sup>	3.93 $\pm$ 0.19 <sup>b</sup>	3.40 $\pm$ 0.13 <sup>b</sup>
Cr root ( $\mu\text{g/g DW}$ )	23.37 $\pm$ 0.36 <sup>a</sup>	0.53 $\pm$ 0.04 <sup>b</sup>	0.74 $\pm$ 0.02 <sup>b</sup>
Co root ( $\mu\text{g/g DW}$ )	3.04 $\pm$ 0.09 <sup>a</sup>	2.38 $\pm$ 0.03 <sup>b</sup>	0.86 $\pm$ 0.01 <sup>c</sup>
Mo root ( $\mu\text{g/g DW}$ )	0.94 $\pm$ 0.05 <sup>a</sup>	0.85 $\pm$ 0.02 <sup>b</sup>	0.46 $\pm$ 0.02 <sup>c</sup>

the Zn supplementation in a concentration-dependent way (Table 1). There is evidence published about the crosstalk of Zn and another elements like Fe, Cu and Cd, but a comprehensive evaluation is still lacking [72]. In *Arabidopsis thaliana* roots, Zn treatment caused decreased Fe and Cu content [72], while in *A. halleri* Zn treatment reduced Ni [86] and Mn [73] content of the roots. In the present experimental system, excess Zn decreased the *in planta* concentrations of relevant

microelements like Fe, Cu, Mo, Co, Cr thus disturbing microelement homeostasis of *B. napus* roots which in turn may contribute to growth reduction.

### 3.2. Distinct tyrosine nitration response associated with different root architectural changes

Protein tyrosine nitration, a posttranslational modification is observed to participate in many physiological and stress-related processes [74] and proved to be a suitable biomarker in case of Zn-stressed *Brassica* species in hydroponics [38,62]. The presence of tyrosine nitration has been proved in control, healthy plants as well [15,38,75]. In the present experimental setup six nitrated protein bands were detectable in the 35-10 kDa molecular weight zone (Fig. 4A) under control circumstances, proving that a physiological nitroproteome is present in soil-grown roots as well. The low and high Zn concentrations caused a diverse response. 10 ppm Zn supplementation caused a tyrosine nitration-pattern rearrangement: the nitration of several protein bands decreased that were nitrated under control conditions, while four newly nitrated protein bands appeared (approximately 250, 50, 45 and 23 kDa) (Fig. 4A, white arrows). In contrast, 500 ppm Zn treatment resulted in a generally increased tyrosine nitration in the lower molecule weight zone (Fig. 4A, same bands as in control roots, black arrows), and also a newly nitrated protein band appeared (approximately 25 kDa) (Fig. 4A). The existence of a basal nitration state of the protein pool is observed in many species (reviewed in Ref. [74]), and previous studies also found that Zn induces increased tyrosine nitration in the



**Fig. 4.** Representative immunoblot showing protein tyrosine nitration in the roots of *B. napus* grown in soil-filled rhizotrons under control circumstances (60 ppm Zn) and Zn supplementation (A). White arrows show newly nitrated protein bands while black arrows show protein bands with increased nitration compared to the control ( $n = 3$ ). (B) Immunolocalisation of 3-nitrotyrosine in root tips of *B. napus* grown in soil-filled rhizotrons under control circumstances and Zn supplementation (bar = 100  $\mu\text{m}$ ). (C) Changes in the fluorescence consistent with peroxynitrite in the root apical meristem of *B. napus* upon Zn treatment. Different letters indicate significant differences according to Duncan-test ( $n = 10\text{--}15$ ,  $P < 0.05$ ).

roots of *B. napus* [38,62]. The pattern and rate of tyrosine nitration however is different in the present study compared to the previous results, possibly because of the different experimental setup (hydroponics vs soil filled rhizotron) and applied Zn concentrations.

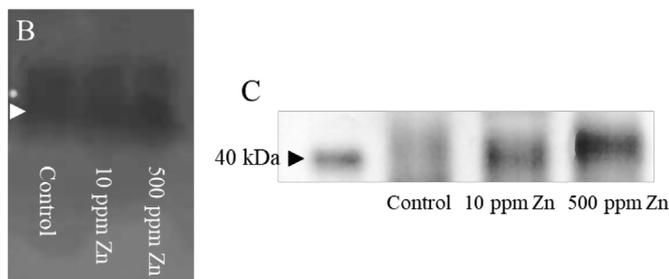
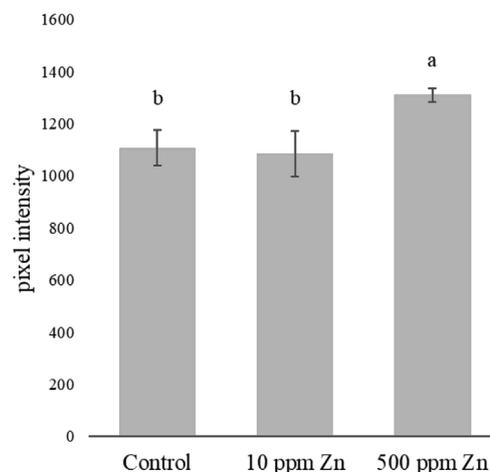
We also detected protein tyrosine nitration *in situ* in the root tips, and the overall strength of the tyrosine nitration-dependent fluorescence showed correlation with the previous results: compared to the control, fluorescence did not increase significantly in the 10 ppm Zn-treated root tips, while it was considerably higher in the 500 ppm Zn-treated roots. Protein tyrosine nitration is a result of a series of changes in the nitro-oxidative signalling network, and the responsible carbonate, hydroxyl radicals and nitrogen dioxide radicals are derived from peroxynitrite ( $\text{ONOO}^-$ ) (reviewed in Ref. [74]). The Zn-triggered changes in fluorescence consistent with  $\text{ONOO}^-$  formation show similar tendencies like the level of tyrosine nitration, since compared to the control, only the 500 ppm Zn treatment induced significant  $\text{ONOO}^-$ -associated fluorescence increment (Fig. 4C), also corroborating tyrosine nitration results.

### 3.3. Distinctive changes in the underlying nitro-oxidative signal transduction network

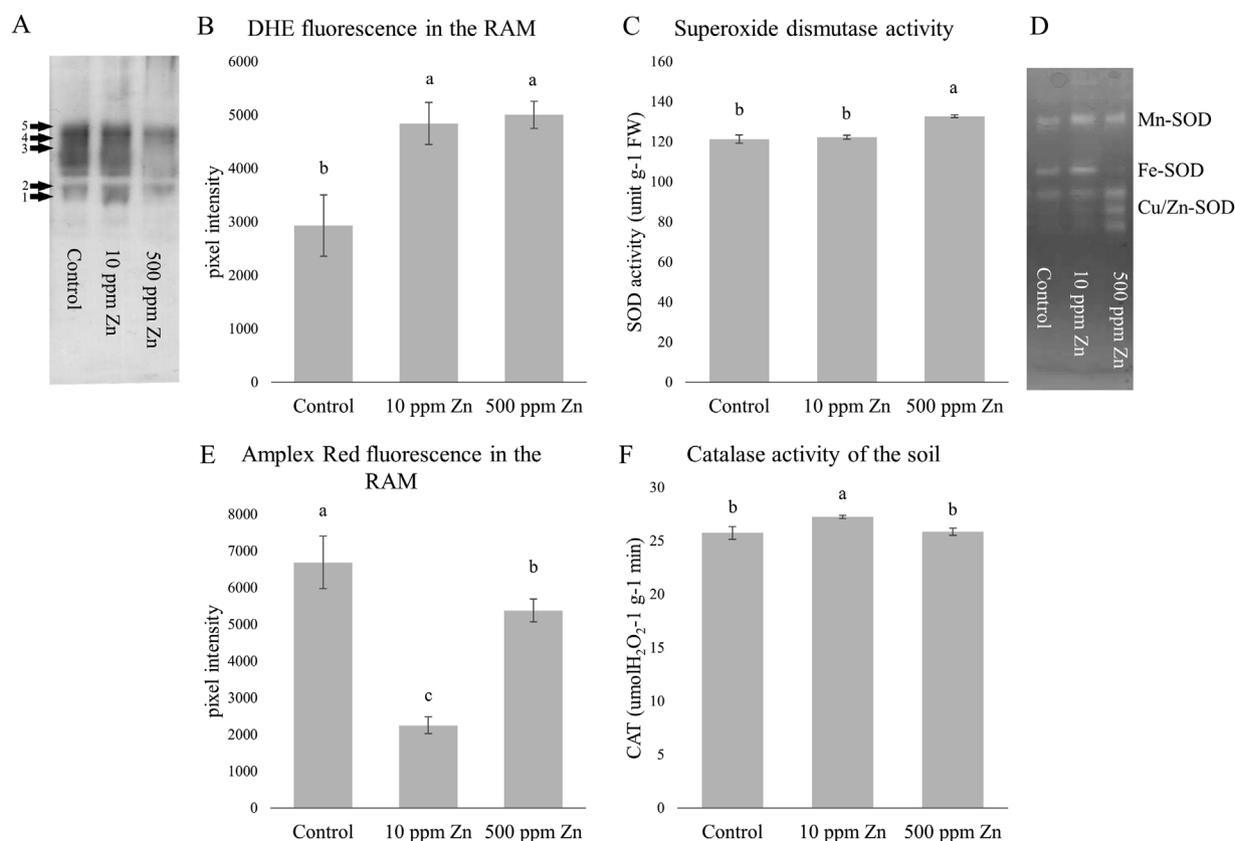
Peroxyntirite is derived from superoxide anion and nitric oxide, thus the amount of  $\text{ONOO}^-$  and ultimately the appearance of tyrosine nitration depends on the production and accumulation of these reactive species [74]. Similar to  $\text{ONOO}^-$ , fluorescence consistent with NO formation in the root apical meristem was increased only by 500 ppm Zn treatment (Fig. 5A). NO can be also stored in the form of S-nitrosoglutathione (GSNO), which can act as a mobile NO reservoir [76]. GSNO can either spontaneously decompose to NO or can be enzymatically reduced by GSNOR to GSSG and  $\text{NH}_3$  [77]. Zn treatment, especially 500 ppm Zn supplementation, increased both GSNOR enzyme activity (Fig. 5B and suppl. fig. 2) and protein amount (Fig. 5C), however the reason behind the size-shift of the immunopositive band is yet unknown. The higher NO-associated fluorescence discussed above can be related to the higher activity and presence of GSNOR, which is responsible for GSNO removal, suggesting that Zn induces a severe disturbance in NO homeostasis in the roots treated with 500 ppm Zn.

Besides the homeostasis of reactive nitrogen species, the balance of reactive oxygen species was also changed by Zn treatment. Both Zn

### A DAF-FM fluorescence in the RAM



**Fig. 5.** Fluorescence consistent with NO formation in the root apical meristem of *B. napus* grown in soil-filled rhizotrons containing optimal (control, 60 ppm total Zn) or supraoptimal (10 ppm and 500 ppm Zn supplementation) Zn concentrations. (A). Different letters indicate significant differences according to Duncan-test ( $n = 10\text{--}15$ ,  $P < 0.05$ ). Representative native-PAGE (6%) of *B. napus* root extracts and staining for GSNOR activity (white arrow) (B). Representative immunoblot showing GSNOR protein abundance in roots of control or Zn-treated *B. napus* (C) ( $n = 3$ ).



**Fig. 6.** (A) Native-PAGE (10%) separation of NADPH oxidase isoenzymes in the root of *B. napus* supplemented with 10 and 500 ppm Zn, compared to the control (60 ppm total Zn). Putative isoenzymes are numbered and indicated by black arrows. (B) Fluorescence consistent with superoxide formation in the root apical meristem. Total superoxide dismutase activity (C) and superoxide dismutase isoenzymes separated on native-PAGE (10%) (D). (E) Fluorescence consistent with H<sub>2</sub>O<sub>2</sub> formation in the root apical meristem of *B. napus* and (F) catalase activity of the soil supplemented with 10 and 500 ppm Zn. Different letters indicate significant differences according to Duncan-test (n = 3 (SOD, CAT) or 10–15 (O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub>), P < 0.05).

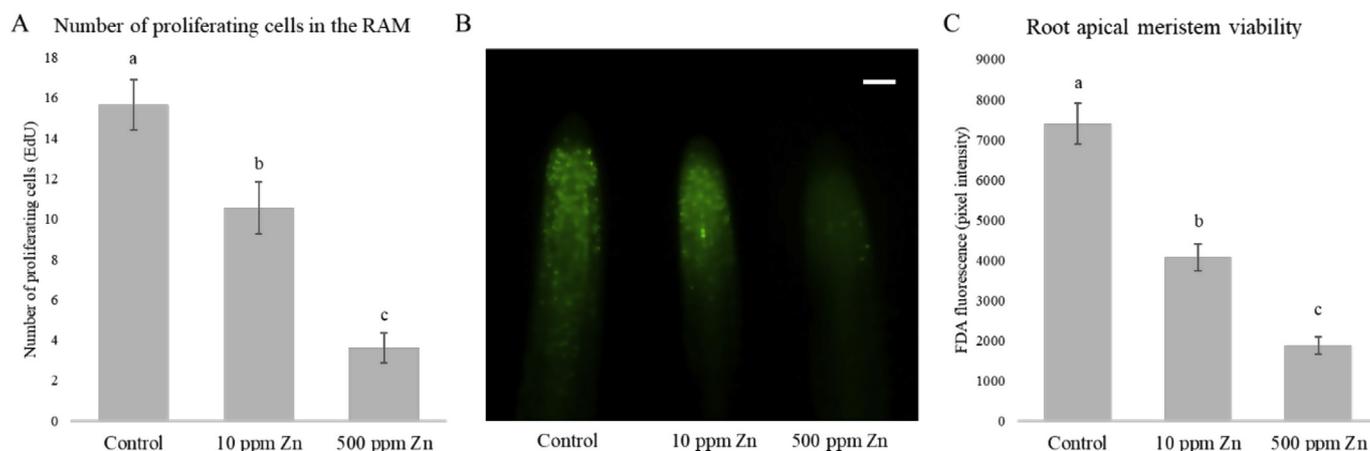
treatment increased the fluorescence consistent with superoxide formation in the root tips, regardless of the applied concentration (Fig. 6B). The native-PAGE analysis of the O<sub>2</sub><sup>-</sup> producing NADPH oxidase enzyme revealed five isoenzymes, and the total activity of this enzyme decreased in a concentration-dependent way (Fig. 6A, for separate isoenzyme activities see suppl. fig. 3), suggesting that there is another source of O<sub>2</sub><sup>-</sup> in the root tips, like plant peroxidases that mainly generate O<sub>2</sub><sup>-</sup> through oxidation of phenolic compounds [78]. Measurement of the SOD activity shown increment only in case of 500 ppm Zn treatment (Fig. 6C), and the native-PAGE analysis of the enzyme identified that there is a rearrangement of isoenzyme activities (Fig. 6D). While the overall SOD activity in case of 10 ppm Zn treatment did not change significantly, in the background a very slight increment in the Mn and Fe-SOD isoenzyme-activity could be detected (Suppl. fig. 4AB). On the other hand, in case of 500 ppm Zn treatment, Fe-SOD isoenzyme activity decreased notably, while the activity of all three Cu/Zn-SOD isoenzymes increased significantly (Suppl. fig. 4C). One of the reasons of the decreased Fe-SOD activity could be the reduced availability of iron as previously shown in Table 1, while the also reduced accessibility of copper did not affect Cu/Zn-SOD activity in a negative way.

Fluorescence consistent with hydrogen peroxide formation in the root tips decreased significantly after Zn treatment, and the different amounts of supplied Zn caused different responses. The H<sub>2</sub>O<sub>2</sub>-associated fluorescence of the root tips was the lowest in case of 10 ppm Zn supplementation (Fig. 6E); however, the activity of SOD in the roots did not explain this difference. Therefore, as an interesting possibility, soil CAT activity was examined. As the effect of Zn supply, the CAT activity in the soil increased significantly in case of 10 ppm Zn supplementation

(Fig. 6F), which was accompanied by the lowest H<sub>2</sub>O<sub>2</sub>-related fluorescence in the root tips, suggesting that somehow the growth medium may be able to buffer/extinguish the produced H<sub>2</sub>O<sub>2</sub> in the root apical meristem. The CAT activity in the soil partly depends on the total number of aerobic heterotrophic bacteria (measured with colony forming unit counting), which was only negatively affected by the 500 ppm Zn treatment, while the addition of 10 ppm Zn slightly increased bacterial counts in the growth media (data not shown). Also, micromycetes, as the members of soil microbial communities often produce extracellular catalases [41], providing a further explanation for decreased fluorescence consistent with H<sub>2</sub>O<sub>2</sub> formation in the root tips. In general, heavy metal contamination can either lower [33] or increase [87]; [37] the enzymatic activities of soil microbial communities, showing the complexity of the heavy metal induced responses of soil enzyme activities [80]. reported that catalase is inhibited by Zn stress, although this inhibition is much less pronounced than invertase or urease activity loss.

### 3.4. Subsequent viability loss of the root tips

Viability of the root apical meristem seriously affects the growth of the root system. The above discussed Zn uptake and Zn-induced changes in the nitro-oxidative homeostasis affects the development of the root system by modifying the viability and proliferation rate of the apical meristem. According to the fluorescent EdU staining, which detects cell DNA synthesis [81], the number of cells with active DNA replication decreased significantly by both Zn treatment (by 33 and 77%, respectively) (Fig. 7A and B). With FDA staining we detected the viability of the root apical meristem, and it showed similar changes as seen

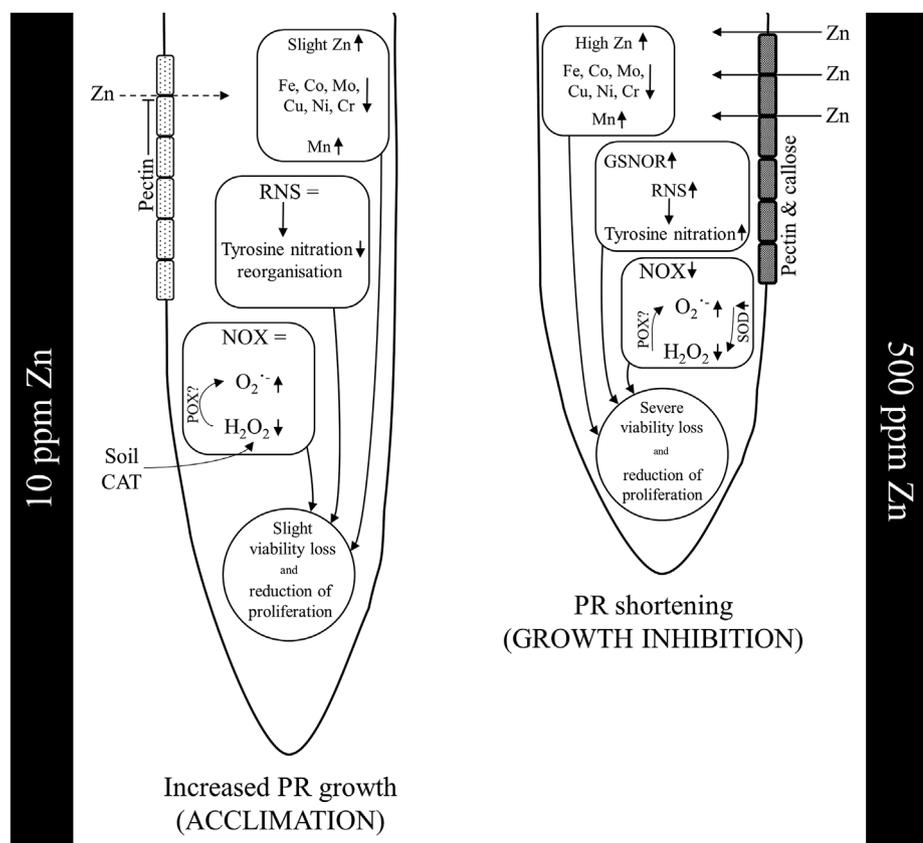


**Fig. 7.** (A) Number of cells with active DNA synthesis in the meristematic zone of the roots of *B. napus* supplemented with 10 or 500 ppm Zn compared to the control (60 ppm total Zn). (B) Representative image of the root tips stained with EdU, showing the number and localisation of cells with active DNA synthesis in the root tips supplemented with 10 or 500 ppm Zn compared to the control (60 ppm total Zn) (bar = 100 μm). (C) Viability of the root apical meristem supplemented with 10 or 500 ppm Zn compared to the control (60 ppm total Zn). Different letters indicate significant differences according to Duncan-test (n = 10–15, P < 0.05).

in the number of proliferating cells, both Zn supplementations caused significant decrease in their viability (by 45 and 75%, respectively, compared to the control, if that's fluorescence is defined by 100%) (Fig. 7C), suggesting that the cells with decreased DNA replication activity correlate closely with the viability of the meristematic cells. These results do not necessarily coincide with the primary root growth data, since besides proliferation and viability, many other factors (alterations in the primary metabolism or changes in the hormonal homeostasis) influence primary root elongation [82].

#### 4. Conclusions

The present study compared the effect of two different Zn supplementation on the rapeseed RSA and the underlying processes (summarised in Fig. 8). The two applied Zn concentrations triggered two completely different growth responses in *B. napus* root system. In the background of the 10 ppm Zn supplementation-induced positive growth response the pattern of tyrosine nitration rearranged significantly, and four new protein bands became nitrated. There were no severe disturbances in the nitro-oxidative signalling network; and due to the low Zn treatment and mild Zn uptake the composition of the cell walls changed only slightly in the root tips (pectin content increment). It has



**Fig. 8.** Schematic model summarising the results presented in this study. 10 ppm Zn supplementation caused a positive growth response with slight Zn uptake and tyrosine nitration reorganisation in the background, while no oxidative or nitrosative stress was detectable. 500 ppm Zn treatment inhibited root growth, and this stress response was accompanied by high Zn uptake and indicated by increased cell wall modifications, tyrosine nitration and fluorescence consistent with ROS/RNS formation. (An upward arrow indicates increase while a downward arrow shows decrease; = means no significant change.)

to be noted though, that despite the positive growth response, the viability of the root apical meristem cells decreased to some extent. On the other hand, 500 ppm Zn supplementation caused severe growth inhibition, what was co-occurred with increased tyrosine nitration. The nitro-oxidative balance was disturbed, both the fluorescence consistent with ROS and RNS formation increased significantly. Due to the high Zn concentration, Zn uptake was high in the root system and it caused severe alterations in the cell walls (both pectin and callose contents increased) and all these processes were coupled with a significant reduction in the viability of the root apical meristem.

Results suggest that Zn in different amounts triggers different root growth responses accompanied by distinct changes in the metabolism of ROS and RNS consequently resulting in alterations in pattern and intensity of protein tyrosine nitration. These suggest that nitrosative processes have an important role in zinc stress-induced root growth responses.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.niox.2019.06.003>.

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