Summary of the Ph.D. thesis

Heavy metal induced nitro-oxidative stress in *Brassica* species

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Introduction

In the past couple of decades heavy metal contamination of the environment has increased radically due to various geological and anthropogenic effects. This has continued to such an extent that the stress caused to plants can no longer be considered negligible. All plants are capable of the uptake and accumulation of essential metals (for example copper, manganese, zinc); however, the uptake of other non-essential metals is also possible. Heavy metal stress – like other stress factors – induces morphological changes in the roots and shoots of the plants (stress-induced morphogenic responses). Since the root is the first structure to encounter the heavy metals (dissolved in the soil solution), it is fundamental to the study of stress responses. Thus, my primary goal was the investigation of the changes in the root system due to heavy metal stress.

Copper (Cu) and zinc (Zn) are essential micronutrients. The former is a redox-active element: it directly induces the formation of reactive oxygen species (ROS) which leads to oxidative stress. In contrast, the latter is a non-redox-active element, causing oxidative stress indirectly by the modulation of antioxidant capacity. At high concentrations both have a negative effect on the viability of the root apical meristem, thereby decreasing root growth, and negatively affecting the growth of the entire plant. However, at appropriate concentrations Zn has a positive effect on root growth, and Cu can stimulate the formation of new meristematic zones and lateral roots. Furthermore, besides their effects on root development, changes in the homeostasis of reactive oxygen (for example hydrogen-peroxide, superoxide radical) and nitrogen species (nitric oxide, peroxynitrite) occur.

Formation of ROS can be linked to a wide range of stress responses. This suggests an important role as intermediates in heavy metal stress and the appearance of stress-induced morphogenic responses, as well as their connections to the signalisation of reactive nitrogen species (RNS). In the process of protein tyrosine nitration induced by RNS, peroxynitrite reacts with tyrosine amino acids, modifying the structure and activity of proteins. Moreover, nitric oxide (NO) has an important role as a mobile signal molecule in root growth and development.

The metabolism of ROS and RNS are connected at various points. The concept of nitro-oxidative stress has only recently become the subject of research in the field of plant

biology. This new research is helping in the characterization of the effects of heavy metals on root development in ways previously unexplored.

During my studies I compared the effects of copper and zinc on Indian mustard (*Brassica juncea* L. Czern.) and oilseed rape (*Brassica napus* L.) plants. Both species are from the *Brassicaceae* (*Cruciferae*) family and have important economical roles, owing to their cultivation as oil plants. Therefore, the investigation of their stress-tolerance – in this case their heavy metal-tolerance - is particularly important. Besides their economical roles, there are initiatives for their application in phytoremediation. Thus, due to the increasing contamination of the environment with heavy metals, the expansion of our knowledge on the heavy metal-tolerance of crop plants is undoubtedly important for the future.

Objectives

During my Ph.D. work, my goals were to compare copper and zinc sensitivity and the effects of these two heavy metals on two economically important *Brassica* species (*Brassica juncea* L. Czern. and *Brassica napus* L.); to investigate oxidative- and nitrosative stress processes, together with the clarification of their role in the development of heavy metal tolerance.

During my research I sought answers to the following questions:

1. How much copper and zinc can the two teste~~d~~ species take up; in which of their organs does it accumulate; and what kind of changes does the copper and zinc treatment cause to their microelement-homeostasis?
2. What kind of morphological changes do copper and zinc cause and does the application of the heavy metal cause the appearance of symptoms of stress-induced morphogenic response?
3. Do the two heavy metals induce any cell wall modifications in the roots of the species; and how does the viability of the root apical meristem change~~s~~ due to copper and zinc stress?
4. Is there any copper- or zinc-induced change in the metabolism of RNS, in their interactions with ROS? Does secondary, nitrosative stress develop~~s~~ in the roots? Is there any difference between the two *Brassica* species with regards the size of nitroproteome?
5. Is there any connection between the copper and zinc-induced nitrosative and oxidative processes and the heavy metal tolerance of the *Brassica* species?

Materials and methods

Plant species and growth conditions

The following experiments were performed on Indian mustard (*Brassica juncea* L. Czern.) and oilseed rape (*Brassica napus* L.) plants. Both species belong~~s~~ to the *Brassicaceae* family, of which there are several ~~an~~other heavy metal tolerant and hyper-accumulator species. Furthermore, these species are capable of producing high amounts of biomass in short periods.

Brassica juncea and *Brassica napus* seeds were surface-sterilized and then placed onto perlite-filled Eppendorf tubes floating on full-strength Hoagland solution. The seedlings were precultivated for nine days – until the appearance of the first leaves – and then the nutrient solution was changed and supplemented with 10, 25 and 50 μM $CuSO_4$ or 50, 150 and 300 μM $ZnSO_4$ for seven days. Control plants were grown in full-strength Hoagland solution containing 0.5 μM $CuSO_4$ and 5 μM $ZnSO_4$. The plants were kept in a greenhouse at a photon flux density of 150 μmol m^{-2} s^{-1} (12/12h light/dark cycle) at a relative humidity of 55-60% and 25±2°C for 7 days.

Element content analysis

The concentrations of microelements were measured by using inductively-coupled plasma mass spectrometry (ICP-MS, Thermo Scientific XSeries II, Asheville, USA). Values of Zn and other microelement concentrations are given in μg/g dry weight (DW).

Morphological measurements

After 7 days of treatment, both the fresh and dry weight (g) of the shoot and root system was measured, together with the length (cm) of the primary roots. The number (pcs/root) of the lateral roots was also counted.

Microscopic investigations

The roots of *Brassica* plants labelled with different fluorophores were investigated under a Zeiss Axiovert 200M inverted microscope (Carl Zeiss, Jena, Germany) equipped with different filter sets, suitable for each of the dyes. 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.

Localisation of zinc within the root tips

For the localisation of zinc within the root tips, two different fluorophores, Zinquin (ethyl (2-methyl-8-p-toluenesulphonamido-6-quinolyloxy)acetate) and Zinpyr-1 (4′,5′-Bis[bis(2-pyridylmethyl)aminomethyl]-2′,7′-dichlorofluorescein) were used.

Localisation of zinc on cellular level, labelling cell walls/dead cells

Cellular-level zinc localisation was performed with the simultaneous use of Zinquin-1 fluorophore and propidium-iodide for labelling cell walls and dead cells; by confocal laser scanning microscope (Olympus LSM 700, Olympus, Tokyo, Japan) using excitation at 488 nm with a 100mW Ar ion laser and a ×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.

Detection of cell wall modifications in roots

Callose deposition in the root tissues was determined by image analysis using aniline blue staining; detection of lignification was performed with phloroglucinol staining, based on the Wiesner-reaction.

Detection of lipid peroxidation in roots

Products of lipid peroxidation (such as malondialdehydes) were visualized using Schiff's reagent.

Measuring of the viability of apical root tip meristems

For the determination of cell viability in the root tips, fluorescein diacetate (FDA) staining was used.

Detection of ROS and RNS

Dihydroethidium (DHE) was used for visualization of superoxide anion contents in the root tips; for hydrogen peroxide detection, root segments were incubated in Ampliflu^{TM} (10-acetyl-3,7-dihydroxyphenoxazine, ADHP or Amplex Red) solution.

The NO levels in *Brassica* root tips were determined by 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA), while for the *in situ* and *in vivo* detection of peroxynitrite ($ONOO^-$), 3'-(*p*-aminophenyl) fluorescein (APF) was applied.

Measuring the activity of the components of enzymatic antioxidant system

Superoxide dismutase (EC 1.15.1.1) activity 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; activity of ascorbate peroxidase (APX; EC 1.11.1.11) was measured by monitoring the decrease of ascorbate content at 265 nm.

Immunoprecipitation, SDS-PAGE and Western blot

Crude extracts from plant material were immunoprecipitated by using Thermo Scientific Pierce Crosslink Magnetic IP/Co-IP Kit (Hudson, NH, USA). The beads were cross-linked with an antibody against 3-nitrotyrosine. After purification, immunoprecipitated samples were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% acrylamide gels. For western blot analysis, proteins were transferred to PVDF membranes using the wet blotting procedure. After transfer, membranes were used for cross-reactivity assays with a rabbit polyclonal antibody against 3-nitrotyrosine diluted 1:2000. Immunodetection was performed by using an 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.

Summary of results

During my Ph.D. studies, I compared the effect of copper and zinc on two economically important plant crop species: *Brassica juncea* and *Brassica napus*. I investigated their responses to heavy metal stress by analysis of changes in their morphology, biochemistry and molecular levels. I performed the investigation of the two species' heavy metal uptake and changes in their microelement homeostasis. Furthermore, I studied the stress-induced morphogenetic responses, as well as the underlying background processes such as changes in the cell walls, loss of root apical meristem viability, changes in the levels of reactive oxygen and nitrogen species and their interconnections.

Based on my results, I can summarise:

1. According to the element content analysis of the two species grown in excess copper or zinc, it can be stated that the two species have demonstrable metal uptake capacity, accumulating the two metals primarily in their root systems. The root-to-shoot transport is more effective with that of zinc than that of copper stress: the plants translocate zinc in an order of magnitude better into their leaves. Both copper and zinc stress caused alterations in the Cu/Zn, Fe and Mn homeostasis in the roots of the two investigated species.
2. The lowest applied copper treatment caused the development of the symptoms of stress-induced morphogenetic responses in both species, while the lowest zinc treatment caused a similar effect only on *B. napus*. It was also found that the root growth of both species is sensitive to copper stress, while only the root growth of *B. napus* proved to be sensitive to excess zinc.
3. The copper and zinc resulted in different cell wall alterations in the roots of the two species. Copper caused lignin formation at the base of the lateral roots, which was proved to be hydrogen peroxide-dependent, while zinc resulted in callose deposition in the cell walls of the root tips. The two applied heavy metals affected the viability of the root apical meristems in different ways. Copper caused significant viability-loss in both species, while zinc excess did not affect the viability of the root tips of *B. juncea*.

Moreover, the decrease in viability experienced in *B. napus* was significantly smaller than in case of copper excess.

4. My results highlighted the copper- and zinc-induced changes in the homeostasis of the reactive oxygen and nitrogen species. Copper caused a slight increase in the NO content of *B. juncea* root tips, whereas the levels decreased in the case of *B. napus* Moreover, while the peroxynitrite level in both species' root tips increased significantly, increase in the protein tyrosine nitration was undetectable. Therefore, despite the strong oxidative stress, no comment can be made regarding nitro-oxidative stress.

   Conversely, zinc increased both the NO and peroxynitrite levels of both species' root tips, and despite the different level of oxidative stress detected, protein tyrosine nitration increased in both species.

5. According to my results, oxidative stress plays a predominant role in the heavy metal sensitivity of the two *Brassica* species. Where oxidative stress was undetectable, the growth of the root was not inhibited, thus the plant tolerated heavy metal stress better (in this case *B. juncea* zinc excess). The results also suggest that the sensitivity of the two species to copper or zinc stress is determined by the level of oxidative stress, rather than by the nitrosative stress processes.

According to the results, it can be concluded that both species were able to survive in a copper- or zinc-rich environment. Moreover, they were able to accumulate these heavy metals in their root system, however it caused severe growth inhibition in most cases. The only exception was *B. juncea* submitted to zinc stress, where neither the viability of the primary root tip meristem, nor the growth of the root system decreased. In summary, both species proved to be moderately copper and zinc tolerant even in the early stages of their growth. In the background there are different organ and cellular level tolerance mechanisms: one option is the exclusion of the heavy metals from the shoot, to minimize the translocation to the shoot. Also the root system exposed to low concentrations of metal showed morphological adaptation, to ensure better water and nutrient uptake and in conclusion better survival. At the cellular level, root cells were trying to exclude heavy metals from their cytoplasm. This was facilitated by lignification in the case of copper stress and callose deposition and

immobilisation of zinc in the root cell walls during zinc stress. In the case of copper stress, this was facilitated by lignification; in that of zinc stress, it was facilitated by callose deposition and zinc immobilisation in the cell walls

However, the changes caused in the ROS and RNS homeostasis in the roots of *B. juncea* which were exposed to the two heavy metals showed similar trends. The fact that zinc did not cause lipid peroxidation (therefore no oxidative stress) is a point of considerable importance. In contrast, in every other case~~s~~ (in both species submitted to copper stress and in *B. napus* submitted to zinc stress) lipid peroxidation (oxidative stress) was detected, which was coupled with the inhibition of root development. Changes in the levels of RNS proved to be species-specific (higher increment in *B. juncea*, while lower in *B. napus*). This did not increase protein tyrosine nitration in copper-stressed plants, whereas zinc stress caused significant increase in protein tyrosine nitration in both species.

Based on the results obtained during my research, it is clear that behind the morphological changes due to heavy metal stress there is a complicated network of changes, from the alterations in the element content to the development of nitrosative and oxidative stress.

In my opinion, with the work that I have done, I have contributed to the better understanding of the development of nitro-oxidative stress due to abiotic (heavy metal) stress.

Publications related to the topic of the thesis **mtmt ID: 10029489**

- **Gábor Feigl**, Devanand Kumar, Nóra Lehotai, Nóra Tugyi, Árpád Molnár, Attila Ördög, Ágnes Szepesi, Katalin Gémes, Gábor Laskay, László Erdei, Zsuzsanna Kolbert (2013) Physiological and morphological responses of the root system of Indian mustard (*Brassica juncea* L. Czern.) and rapeseed (*Brassica napus* L.) to copper stress.

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- **Gábor Feigl**, Nóra Lehotai, Árpád Molnár, Attila Ördög, Marta Rodríguez-Ruiz, José M. Palma, Francisco J. Corpas, László Erdei, Zsuzsanna Kolbert (2015) 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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- **Gábor Feigl**, Devanand Kumar, Andrea Pető, Nóra Lehotai, Ágnes Szepesi, László Erdei, Zsuzsanna Kolbert (2012) Studying the effect of copper in *Brassica juncea* and *Brassica napus* root tips: metabolism of reactive oxygen and nitrogen species and morphological adaptation.

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- **Gábor Feigl**, Devanand Kumar, Andrea Pető, Nóra Lehotai, Attila Ördög, Árpád Molnár, Zsuzsanna Kolbert, László Erdei (2012) The effect of zinc on the microelement homeostasis and the metabolism of reactive signal molecules in *Brassica juncea* and *Brassica napus*.

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- Zsuzsanna Kolbert, Andrea Pető, Nóra Lehotai, **Gábor Feigl**, Attila Ördög, László Erdei (2012) In vivo and in vitro studies on fluorophore-specificity.

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- Zsuzsanna Kolbert, Andrea Pető, Nóra Lehotai, **Gábor Feigl**, László Erdei (2015) Copper sensitivity of nia1nia2noa1-2 mutant is associated with its low nitric oxide (NO) level.

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Biometals (IF: 2.503) submitted

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Plant and Soil (IF: 3.235) submitted